Investigation of the anti-cancer effects of rosemary *(Rosmarinus Officinalis L.)* extract in human breast and prostate cancer cells

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Abstract

Breast and prostate cancer are the most frequently diagnosed cancers in women and men respetively, in North America. Triple-negative breast cancer (TNBC) cells do not express estrogen receptor, progesterone receptor, and human epidermal growth factor receptor-2 (HER-2). TNBC accounts for 15% of all breast cancer cases, is aggressive in nature, and is characterized by resistance to chemo and radiotherapy thus, finding new approaches to inhibit it are urgently needed. Similarly, prostate cancer is typically characterized by the expression of androgen receptor (AR) and prostate-specific antigen (PSA). Prostate cancer that is AR positive can be treated with hormonal therapy. In contrast, AR negative prostate cancer is more aggressive and does not respond to hormone therapy, thus new approaches, including identifying specific signaling molecules that are overactivated and could be targeted, are required to effectively treat this subtype of prostate cancer.

Rosemary extract (RE) has been shown to have anti-cancer properties *in vitro* and *in vivo*. However, limited evidence exists regarding its effect on triple-negative breast cancer and AR negative prostate cancer. In this study, we examined the effects of RE on triple-negative breast cancer cell (MDA-MB-231) and androgen insensitive prostate cancer cell (PC-3) proliferation, survival/apoptosis, and migration. In addition, we investigated the effect of RE treatment on key signaling molecules involved in cancer cell proliferation and survival.

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AMPK: 5’ adenosine monophosphate-activated kinase

AR: androgen receptor

ATP: adenosine triphosphate

CA: carnosic acid

COH: carnosol

DHT: dihydrotestosterone

DTX: docetaxel

DMBA: 7,12-Dimethylbenz(*a*)anthracene

DNA: deoxyribonucleic acid

ECM: extracellular matrix

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

ER: estrogen receptor

ERα: estrogen receptor alpha

ER+: estrogen receptor positive

ER-: estrogen receptor negative

ERK: extracellular signal regulated kinase

FADD: fas associated protein with death domain

Fas: fibroblast associated antigen

FGFR-2: fibroblast growth factor receptor-2

GAP: GTPase-activating proteins

GDP: guanosine diphosphate

GEF: guanine nucleotide exchange factor

Grb2: growth factor receptor-bound protein 2

GTP: guanosine triphosphate

HER1: human EGFR

HER-2: human epidermal growth factor receptor-2

IHC: immunohistochemistry

JAK: janus kinase

LH: luteinizing hormone

LKB1: liver kinase B1

LHRH: luteinizing hormone-releasing hormone

MAPK: mitogen activated protein kinases

MAPKK or MEK1/2: mitogen-activated protein kinase kinase

MAPKKK: mitogen-activated protein kinase kinase kinase

Met: metformin

MMC: mitomycin-C

MMP: matrix metalloproteinases

mTOR: mechanistic target of rapamycin

mTORC1: mTOR complex 1

mTORC2: mTOR complex 2

NF1: neurofibromin 1

NSCLC: non-small cell lung cancer

p70S6K: p70S6 kinase

PARP: poly ADP ribose polymerase

PH: pleckstrin homology

PI3K: phosphatidylinositol 3-kinase

PIP2: phosphatidylinositol-4,5-bisphosphate

PIP3: phosphatidylinositol-3,4,5-trisphosphate

PKB: protein kinase B

PR: progesterone receptor

PSA: prostate specific antigen

PTB: phosphotyrosine binding

PTEN: phosphatase and tensin homologue

PTX: paclitaxel

RA: rosmarinic acid

RAPTOR: regulatory-associated protein of mTOR

Ras: rat sarcoma

RE: rosemary extract

RICTOR: rapamycin-insensitive companion of mTOR

RTK: receptor tyrosine kinase

SFRE: supercritical fluid rosemary extract

SH2: Src-homology 2

SOS: sons of sevenless

STAT: signal transducers and activators of transcription

T: testosterone

TK: tyrosine kinase

TN: triple negative

TNBC: triple negative breast cancer

TNFα: tumor necrosis factor

TSC1: tuberous sclerosis complex 1

TSC2: tuberous sclerosis complex 2

VEGF-A: vascular endothelial growth factor-A

Chapter 1: Literature Review

1.1 Cancer

Cancer is currently the leading cause of death in developed countries, accounting for an estimated 18.1 million new cases and 9.6 million deaths worldwide in 2018 [1]. The adoption of negative lifestyle behaviours such as smoking, physical inactivity, poor diet, and nutrient overload, have all further increased the global burden of cancer [2].

Cancer is driven by genetic and epigenetic modifications that lead to increased survival and proliferation of cancer cells, while evading apoptosis [3]. This increase in cell proliferation and survival is caused by modulation of signaling pathways that regulate these processes [3]. Mutations that convert proto-oncogenes to oncogenes cause an overactivation of signaling pathways resulting in enhanced proliferation and survival, together with the inhibition/down regulation of tumor suppressor proteins leads to carcinogenesis [3]. Most cancers are epithelial in origin, manifesting as carcinomas in organs such as the lungs, skin, breast, liver, prostate, and pancreas [3]. Sarcomas are mesenchymal in origin, occurring in fibroblasts, myocytes, adipocytes, and osteoblasts [3]. Other types of cancers which are not epithelial in origin can develop in the nervous system or hematopoietic tissue.

Cancer cells acquire key changes in their biological capabilities leading to the development of human tumors [4]. These capabilities enable growth and metastatic potential of cancer cells [4]. The ten recognized hallmark capabilities of cancer are sustaining proliferative signaling, evading growth suppressors, activating invasion/metastasis, enabling replicative immortality, inducing angiogenesis, tumor-promoting inflammation, avoiding the immune system, deregulating cellular energetics, genome instability/mutation and resisting cell death (Figure 1)

[4]. Possibly the most fundamental trait of cancer cells is their ability to sustain their hyper-proliferative capability [4]. Normal cells control the production and release of growth-promoting signals, thereby maintaining homeostasis. Cancer cells deregulate these signals by increasing growth factor production and/or growth factor signaling. Growth factor receptors are cell-surface receptors that contain intracellular tyrosine kinase domains [3–5]. These receptors proceed to increase the activation of signaling pathways that regulate cell growth. The mechanisms by which cancer cells acquire the ability to sustain proliferation can occur by the cell producing growth factor ligands themselves, leading to autocrine proliferative stimulation [4]. In normal cells, cell-to-cell contact as a result of dense cell populations will suppress further proliferation, whereas this feature of contact inhibition is abolished in cancer cells [4]. Cancer cells can also sustain proliferation by increasing the levels of receptor proteins displayed on the cell's surface, which will cause the cell to become hyper-responsive to growth factor ligands [4]. These are the most commonly mutated/hyperactive receptors.

In order to maintain their proliferative capabilities cancer cells will also evade signals from genes that inhibit growth-stimulation. These tumor suppressor genes operate to limit cell growth and activate senescence. Cancer cells will evade apoptosis, which is a programmed cell death that serves as a barrier to cancer development [4]. Apoptosis is controlled by two major pathways; one of which is responsible for receiving and processing extracellular death-receptor signaling (extrinsic pathway) and the other which is responsible for sensing intracellular signals (intrinsic pathway) [4]. Both pathways activate effector caspases that result in cellular disassembly [4]. The extrinsic pathway is triggered by the binding of Fas plasma-membrane death receptors with its extracellular ligand Fas-L [4,6]. When bound, a death complex is formed and recruits Fas associated death domain-containing protein (FADD) and pro-caspase 8 [6]. This

leads to pro-caspase 3 activation, which is the main enzyme responsible for apoptosis. The intrinsic pathway, also known as the mitochondrial pathway, will cause outer mitochondrial membranes to become permeable to cytochrome c in the presence of intracellular signals [4,6,7]. Cytochrome c is released in the cytosol and recruits pro-caspase 9, which activates downstream pro-caspase 3 and leads to apoptosis [4,6,7]. The balance between pro- and anti-apoptotic members of the Bcl-2 family of proteins plays a role in overall apoptosis/survival [4]. The Bcl-2 family is comprised of pro-apoptotic members such as Bax, Bak, and Bad, and anti-apoptotic members such as Bcl-2, Bcl-w and Bcl-X_L [4,6,7]. Following a death signal, pro-apoptotic proteins undergo a post-translational modification that leads to their activation and translocation from the mitochondria, which allows apoptosis to occur [6]. Another key hallmark of cancer cells is their ability to induce angiogenesis. Angiogenesis is the sprouting of new blood vessels, which allows cancer cells to sustain nutrients and oxygen availability [4]. Vascular endothelial growth factor-A (VEGF-A) is a angiogenesis inducer that is involved in the development of new blood vessels during embryonic and postnatal development and induces its effects in target cells/tissues by activating three tyrosine kinase receptors (VEGFR-1-3) [4].

Cancer cells are characterized by their ability to proliferate uncontrollably and evade apoptosis [4,8]. These characteristics are often acquired as a result of mutations in key proteins involved in the signaling pathways responsible for regulating celluar function and maintaining homeostasis [5,9–15]. Molecular signaling pathways of growth factor receptors; such as Epidermal Growth Factor (EGF) Receptor (EGFR) initiate signal transduction pathways such as the phosphatidylinositol 3-kinase (PI3K)/Akt and the Ras/mitogen-activated protein kinase (MAPK) pathway, which can lead to increase cell proliferation and survival [5,9,10,13,16–20]. Oncogenic mutations cause affected genes to be amplified and/or produce mutated proteins with

dysregulated activity [3]. Some of the commonly mutated proteins are growth factor receptor tyrosine kinases (RTKs-such as epidermal growth factor receptors, EGFR), GTPases (Ras), serine/threonine kinases (Raf and Akt), lipid kinases (phosphoinositide 3-kinases, PI3Ks), cytoplasmic tyrosine kinases (Src) and nuclear receptors (estrogen receptor, ER) [3]. Other mutations result in inactivation of proteins that serve as tumor suppressors. The most commonly mutated gene in cancer is the tumor suppressor gene p53 [3,21]. p53 controls cell proliferation and stress signals such as apoptosis and DNA damage responses and its loss/down regulation results in enhanced proliferation and inhibition/suppression of apoptosis [3].

Cancer cells are characterized by their ability to alter their metabolic capabilities to maintain long-term growth, proliferation, and survival, by altering key signaling pathways involved in metabolism and survival processes [3,22,23]. This altered metabolism allows for the increased uptake of glucose and the production of lactate [22]. Both normal and cancer cells obtain their energy from glucose however the processes employed to generate adenosine triphosphate (ATP) markedly differ between these phenotypes. Normal cells undergo aerobic respiration involving cytoplasmic glycolysis and mitochondrial oxidative phosphorylation in conditions with oxygen availability while reverting to glycolysis in low-oxygen states [24,25]. However, cancer cells preferably metabolize glucose through glycolysis and produce lactate regardless of oxygen levels [24,25]. This phenomenon, known as the Warburg Effect, was first observed by Otto Warburg in the 1920s [22]. In tumors and proliferating cells, the rate of glucose uptake and the production of lactate increases, even with a sufficient oxygen supply and fully functioning mitochondria [22]. Lactate secretion increases the acidity of the tumor microenvironment, which can drive cell proliferation and modulate tumor progression [22,26]. The rate of glycolysis and glucose uptake are increased in cancer cells to maintain the increased need for energy production and

compensate for the inefficient energy producing pathway [27,28]. The process of glucose uptake is thereby crucial in driving forward the process of glycolysis and maintaining an adequate supply of energy to the cell [29]. Therefore, the increased cancer cell demand for glucose is compensated by an increased rate of uptake into cell. The ATP yield from glycolysis is much lower than the amount obtained through mitochondrial oxidation however, glycolysis occurs at a 10-100-time faster rate [22].

Figure 1. Acquired capabilities of cancer cells.

1.2 Breast Cancer

Breast cancer is the most frequently diagnosed cancer in women, accounting for roughly 2.1 million newly diagnosed cases and 626,700 deaths worldwide in 2018 [1]. Of these diagnosed cases, it is estimated that over 15% will display the triple-negative phenotype [30,31]. Most triple negative (TN) breast cancers (TNBCs) will be basal-like in gene expression, which exhibits a unique profile and characteristics. TN breast cancer is aggressive, shows an early pattern of metastasis, does not respond to hormone therapy, and as a result limited treatment options are available [10,30]. Additionally, TN breast cancer tends to relapse more frequently and have the worst prognosis of all breast cancer subtypes, this is attributable to the aggressive nature of the disease and the lack of reconigzed molecular targets for therapy [32]. Studies have shown that patients withTNBCs have a much higher death rate than patients with any other breast cancer subtype [33], alongside an incidence rate that is two to three times higher in African-American women [31,34].

In a clinical setting breast cancer is divided into subtypes based on mutations/cancer biology, this classification serves both prognostic and therapeutic significance. Typically, breast cancer will exhibit the expression of estrogen receptor (ER), progesterone receptor (PR) and an amplification of HER-2, as determined by immunohistochemistry (IHC) [35,36]. These markers allow for breast cancer tumors to be classified as hormone receptor positive, HER-2 amplification tumors, or as TNBCs, which do not express ER, PR, and do not have HER-2 amplification [35]. Tumors that express hormone receptors are generally treated with agents that interfere with hormone production or inhibit ER signaling [35,37]. These tumors tend to have a more favourable outcome, when compared to tumors with HER-2 amplification or TNBCs [35]. Tumors that express HER-2 amplification are treated with agents that are tyrosine kinase

inhibitors, such as trastuzumab-based therapy [35,36].

In estrogen receptor positive breast cancer, the binding of the hormone to its receptor leads to downstream activation of the Ras/MAPK and PI3K/Akt pathways [9,37–39] and therefore estrogen receptor signaling has a huge impact on the progression of this subset of breast cancer. Similarly, studies have found that dysregulation of the human epidermal growth factor-2 receptor in breast cancer cells promotes the sequestration of estrogen receptor, leads to downstream activation of the Ras/MAPK cascades and enhances proliferation [37–39]. Despite the absence of these receptors in TNBCs the signaling pathways that regulate cell survival and proliferation remain in an overactivated state. Due to the fact that TN breast cancers do not express these receptors there are no targeted therapies specifically for this sub-class of breast cancer [30]. Studies have shown that while triple negative breast cancers may respond well to primary chemotherapeutic agents such as taxane-based therapies, there is a high risk of relapse if the tumor is not eradicated [30].

The current treatment strategies for breast cancer are surgery, radiotherapy, and chemotherapy. Radiotherapy is often given to TNBC patients following a mastectomy or conservative breast surgery [36]. Specific adjuvant treatments are not very effective in TNBC so polychemotherapeutic agents, such as those offered to high-risk patients are often used and are most effective [36]. The most frequently used first-line treatment for TNBCs are taxane-based therapies [40] and currently therapies targeting apoptotic proteins, EGFR, fibroblast growth factor receptor-2 (FGFR-2), vascular endothelial growth factor (VEGF), and mTOR (mechanistic target of rapamycin), are being developed [36]. Also, the identification of molecular biomarkers in TNBCs is crucial in improving treatment strategies and patient prognosis, since this type of cancer is highly aggressive and difficult to treat due to a lack of targeted therapies [36].

1.3 Prostate Cancer

Prostate cancer accounted for 1.3 million new cases and roughly 359,000 deaths in 2018 globally [1]. It is the most frequently diagnosed cancer in 105 countries, including Canada, the United States and Australia [1,2]. It has been noted that mortality from prostate cancer has increased over the course of the past two decades and is the leading cause of death among men in 46 countries particularily in Sub-Saharan Africa and the Carribean [1,2].

Prostate cancer occurs when the cellular homeostasis of the prostate is disrupted [41]. Androgens are an important growth factor for normal prostate cells, and androgen receptor (AR) is typically expressed in the stromal and epithelial compartments of these cells [41,42]. AR is a member of the steroid hormone receptor family. The growth of prostate cells are dependent on the expression and function of this receptor [41,43]. Production of luteinizing hormone (LH)-releasing hormone (LHRH) by the hypothalamus induces the production of LH by the pituitary [44]. Hypothalamic LHRH production is inhibited when ligands bind to ER, PR, or AR receptors [44]. Androgenic activation of the AR leads to transcriptional activity of AR target genes which are involved in various biological processes such as proliferation and apoptosis [41,43]. When activated by androgenic ligands, testosterone (T) and dihydrotestosterone (DHT), androgen receptors cause the dimerization and translocation of the ligand-receptor complex to the nucleus and act as a transcription factor for induction of different genes [42,43]. Alternatively, the AR complex can lead to the activation of the PI3K/Akt and MAPK pathways [42]. Therefore, AR signaling is directly involved in maintining the balance between proliferation and survival/apoptosis of prostate cells [41]. Aberrant AR expression, activation of oncogenes, and the impairment of tumor suppressor genes, specifically p53, p27, and phosphatase and tensin homologue (PTEN), contribute to the development of prostatic tumors [45–47].

Prostate-specific antigen (PSA) is a serine protease, is synthesized in healthy prostate tissue, benign tumors, and prostate cancer tumors of all grades/stages [48]. PSA concentrations range from roughly 0.3-3 mg/mL [49]. In prostate cancer it is released in the circulatory system and increases blood PSA levels up to a 100,000 fold can be seen [48]. PSA testing has become a marker for initial prostate cancer diagnosis and monitoring treatment response [48]. Men with metastatic prostate cancer will have higher PSA levels compared to localized prostate cancer and patients with higher PSA levels at the time of initial diagnosis have an increased risk of reoccurrence [48]. However, recent evidence has shown that in high-grade prostate cancer, PSA levels <4 ng/mL have been reported [48,50] and up to 10% of prostate tumors produce very trace amounts of PSA [50]. Thus, low PSA levels in high grade prostate cancer (fast growing and likely to spread) may be indicative of differentiation and a poor prognosis [50].

The current treatment strategies for prostate cancer include surgery, radiotherapy, and chemotherapy [51]. Patients with localized prostate cancer are most often treated with radical prostatectomy or radical radiotherapy, however advanced and metastatic prostate cancer is treated with hormonal therapy [51]. Common hormonal therapies often use androgen-receptor inhibitors or LHRH agonists (such as leuprolide, goserelin, buserelin, or nafarelin) that initially increase testosterone production, but with prolonged exposure downregulate the LHRH receptor and inhibit testosterone production [44]. LHRH antagonists (such as cetrorelix, abarelix, or orgalutran) directly inhibit LHRH, which decreases testosterone production [44]. Surgical castration can also decrease T levels by removing the source of production. Many patients do not respond to androgen therapies and therefore, cytotoxic chemotherapeutic agents, such as etoposide, doxorubicin, paclitaxel, and docetaxel as are used as combination therapies for prostate cancer [44].

1.4 Cancer Cell Enhanced Proliferation

Cancer is a complex and multifactorial disease that often involves the over-expression of receptors, growth factors, oncogene activations, and the inactivation of tumor suppressor genes [52]. Epidermal growth factor receptors (EGFRs) are frequently mutated/over-expressed in cancer. The EGFR belongs to the ErbB family of tyrosine kinase receptors (RTK) [23]. Their physiological role is to regulate epithelial tissue development and maintain homeostasis [53]. However, primarily in breast and lung cancer EGFR is a driver of tumorigenesis [53]. The ErbB family of RTKs is composed of four receptors; the EGFR (also known as ErbB-1/HER-1), ErbB-2 (neu, HER-2), ErbB-3 (HER-3), and ErbB-4 (HER-2) [23,52]. These transmembrane glycoproteins have molecular weights ranging from 170-185 kDa [52].

All proteins in this family contain an extracellular ligand-binding domain, a hydrophobic transmembrane domain, and cytoplasmic tyrosine kinase-containing domain [23,52]. ErbB receptors can be activated by binding to ligands of the EGF-family that are produced by cells that express these receptors (autocrine signaling) or by surrounding cells (paracrine signaling) [23]. EGFRs are activated by the binding of a ligand to the extracellular domain of these receptors and induce the formation of heterodimers, which activate the kinase domain, thereby triggering the signaling cascade [23,53]. Receptor activation will lead to the auto-phosphorylation of tyrosine residues within the cytoplasmic tail [23]. These residues then serve as docking sites for proteins containing Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains of intracellular effector proteins [23,52]. These effector proteins will lead to the activation of signaling cascades such as the Ras/MAPK pathway, PI3K/Akt pathway, the anti-apoptotic Akt pathway, and the JAK/STAT signaling pathway, which are involved in cell proliferation, angiogenesis, migration,

survival, and adhesion [52]. In malignant cells these pathways are often dysregulated due to the occurance of mutations in various genes involved in these pathways [52,54].

1.5 PI3K-Akt/PKB

Overactivation of the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B) pathway plays a key role in tumorigenesis [55,56]. PI3K is a lipid kinase that causes phosphorylation of membrane phospholipids on the 3-position resulting in the generation of phosphatidylinositol-3,4,5-triphosphate (PIP3), which contributes to activation of downstream targets such as the serine-threonine protein kinase Akt [57]. PI3K is a heterodimer consisting of a p85 regulatory subunit and p110 catalytic subunit [57,58]. In response to RTK activation, PI3K is recruited to the membrane through the interaction of the Src-homology 2 (SH2) domain of the p85 subunit to tyrosine phosphorylated residues of the activated receptor or to proteins associated with the receptor [57,58]. Ras, a GTPase can also activate PI3K by binding directly to the p110 subunit [57]. At the membrane PI3K will convert phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 will then recruit other downstream molecules by binding to their pleckstrin-homology (PH) domains [57]. PIP3 levels are highly regulated and the lipid phosphatase PTEN acts to rapidly remove it by converting PIP3 back to PIP2, thereby suppressing the PI3K pathway [57]. PTEN is a major tumor suppressor gene and a loss of function mutation of PTEN is highly common in both breast and prostate cancers [57]. Gene amplification of the p110 subunit occurs in ovarian cancer and amplification of Akt is found in ovarian, breast, and colon cancer [57]. Studies analyzing human tumors have shown that at least 33% of TN breast cancers have dysregulated PI3K/Akt pathways and 11.3-35% have a mutation/loss-of-function of the PTEN gene [59,60]. Inactivation of PTEN

by mutation or deletion is identified in roughly 20% of primary prostate tumors and in as many as 50% of castration-resistant tumors [61]. Additionally, Akt over-expression is associated with increased resistance to chemotherapeutic agents such as cisplatin, methotrexate, or paclitaxel [62,63]. Due to the importance of these signaling proteins, several small molecules that target/inhibit Akt are currently in clinical development [64–67].

1.5.1 mTOR

The 289 kDa serine/threonine kinase mechanistic target of rapamycin (mTOR), is a downstream effector of the PI3K/Akt pathway, found in two complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [68]. mTORC1 is sensitive to rapamycin, is involved in mRNA translation and is activated by signaling pathways such as PI3K, MAPK, and 5' adenosine monophosphate-activated kinase (AMPK), in order to control cell growth and survival [68]. mTORC1contains a regulatory associated protein known as RAPTOR, which regulates its activity and recruits mTORC1 substrates [68–71]. mTORC2 is not sensitive to rapamycin or energy signals, but rather regulates the actin cytoskeleton [68,72]. mTORC2 contains Rictor, which is a rapamycin-insensitive companion of mTOR [71,73]. The serine/threonine kinase mTOR is highly involved in the control of cell growth in mammalian cells, the PI3K pathway activates mTOR to stimulate cell growth [74]. Several tumor suppressors such as tuberous sclerosis complex 1 (TSC1 or hamartin), tuberous sclerosis complex 2 (TSC2 or tuberin), and serine/threonine protein kinase 11/liver kinase B1 (LKB1) can inhibit mTOR signaling [74]. Thus, the inactivation of TSC1, TSC2, LKB1, and PTEN play a key role in the development of cancer. The serine/threonine kinase p70S6K is a well-known downstream target of mTORC1. Activated mTORC1 phosphorylates p70S6K, which phosphorylates S6 and leads to mRNA

translation [68]. Many upstream elements can lead to alterations in mTOR expression and can have major effects on tumor progression. As a result mTOR is an appealing target for chemotherapeutic agents and mTOR inhibitors such as sirolimus, deforolimus, everolimus, and temsirolimus are used as a monotherapy or combined therapy for various types of cancers [62,68,75].

1.6 Ras-MAPK

Ras is a human oncogene that was first reported in cancer over three decades ago, it regulates physiological processes such as cell proliferation and survival [76]. Ras GTPase cycles between the active state when bound to guanosine triphosphate (GTP) by guanine nucleotide exchange factors (GEFs) and the inactive state when bound to guanosine diphosphate (GDP) by GTPase-activating proteins (GAPs) that catalyze GTP hydrolysis [15]. When in the active state, GTP bound Ras can activate downstream effectors such as the PI3K and the mitogen-activated protein kinase (MAPK) pathways [15].

Receptor tyrosine kinase (RTK) activation by ligand binding leads to dimerization and autophosphorylation of tyrosine sites in their cytoplasmic domains [19,77]. The phosphorylated tyrosine residues create binding sites for the Src homology 2 (SH2) domain on growth factor receptor binding protein 2 (Grb2) [77]. Grb2 is associated with SOS (sons-of-sevenless), a set of genes encoding GEFs, that exchange GDP for GTP to increase Ras-GTP levels [77]. Activated Ras will then signal its downstream effectors such as the Raf-1 serine/threonine kinase [77]. Raf will then activate two MAPK kinases (MEK1 and MEK2), which will phosphorylate extracellular signal regulated kinases (ERKs) [77]. Activated MAPKs translocate to the nucleus and activate various substrates that regulate protein synthesis.

Mutated Ras is a driver of tumor initiation [15]. There are three human Ras genes (*K-Ras*, *N-Ras*, and *H-Ras*) that encode 4 Ras proteins [15,78]. Overactivation of Ras can occur in tumors without a Ras mutatation by the loss of function of the tumor suppressor neurofibromin 1 (NF1), which encodes a GAP for Ras [15]. Loss of function of this tumor suppressor specifically results in an accumulation of activated GTP bound Ras and decreased GTP hydrolysis [15].

Mitogen-activated protein kinase (MAPK) pathways control cell growth, proliferation, differentiation, migration, and apoptosis [14]. They are comprised of three-tier kinases where by a MAPK is activated upon phosphorylation of a mitogen-activated protein kinase kinase (MAPKK), which is activated upon phosphorylation of a MAPKKK [14].

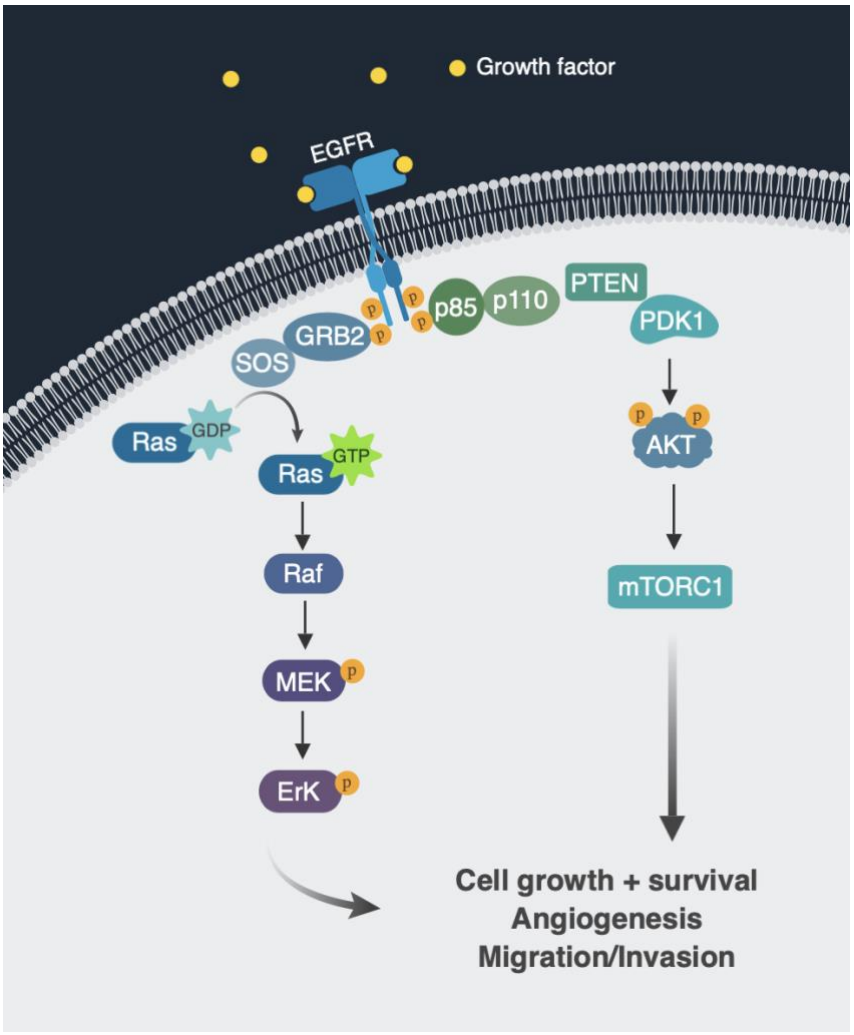


Figure 2. Cell proliferation and survival pathways. The PI3K/Akt and Ras/MAPK pathways play a significant role in cancer and are involved in regulating cell proliferation, survival, apoptosis, and migration.

1.7 Cancer Cell Evasion of Apoptosis

Apoptosis is controlled by two pathways, an extrinsic pathway, responsible for receiving and processing extracellular death-receptor signaling and an intrinsic pathway, responsible for sensing intracellular signals [4]. Both pathways will activate effector caspases that promote cellular disassembly, and are made up of upstream regulator and downstream effector molecules [4]. Caspases are cysteinyl-aspartate proteases, that are initially synthesized as pro-caspases and

cleaved upon activation. The extrinsic pathway involves plasma-membrane bound death receptors with its extracellular ligand Fas-L [4,6]. When bound, a death complex is formed and will recruit Fas associated death domain-containing protein (FADD) and caspase 8 [6]. Caspase 8 will then activate the downstream effector caspase 3, which is the main enzyme responsible for apoptosis. The intrinsic pathway does not involve receptors, but instead will cause outer mitochondrial membranes to become permeable to cytochrome c in the presence of intracellular signals [4,6,7]. Cytochrome c is released in the cytosol and will recruit pro-caspase 9, which activates downstream caspase 3 to execute apoptosis [4,6,7]. The trigger that controls apoptotic signaling is the balance between pro- and anti-apoptotic members of the Bcl-2 family of proteins [4]. The Bcl-2 family is comprised of pro-apoptotic members such as Bax, Bak, and Bad, and anti-apoptotic members such as Bcl-2, Bcl-w and Bcl-X_L [4,6,7]. Following a death signal, pro-apoptotic proteins undergo a post-translational modification that leads to their activation and translocation from the mitochondria so that apoptosis can occur [6].

The poly ADP-ribose polymerase (PARP) family of proteins play a key role in cell apoptosis. PARP is a known substrate of caspases, and it has been shown that caspase 3 and caspase 7 can cleave PARP [79]. PARP is cleaved into fragments by caspase 3/7 which inactivates the enzyme by destroying its ability to respond to DNA strand breaks [80]. The cleavage of PARP by caspases results in the formation of an 89 kDa catalytic fragment and a 24 kDa fragment. PARP helps cells maintain their viability while cleaved PARP is a known indicator of cell apoptosis, as it promotes cellular disassembly [81–83].

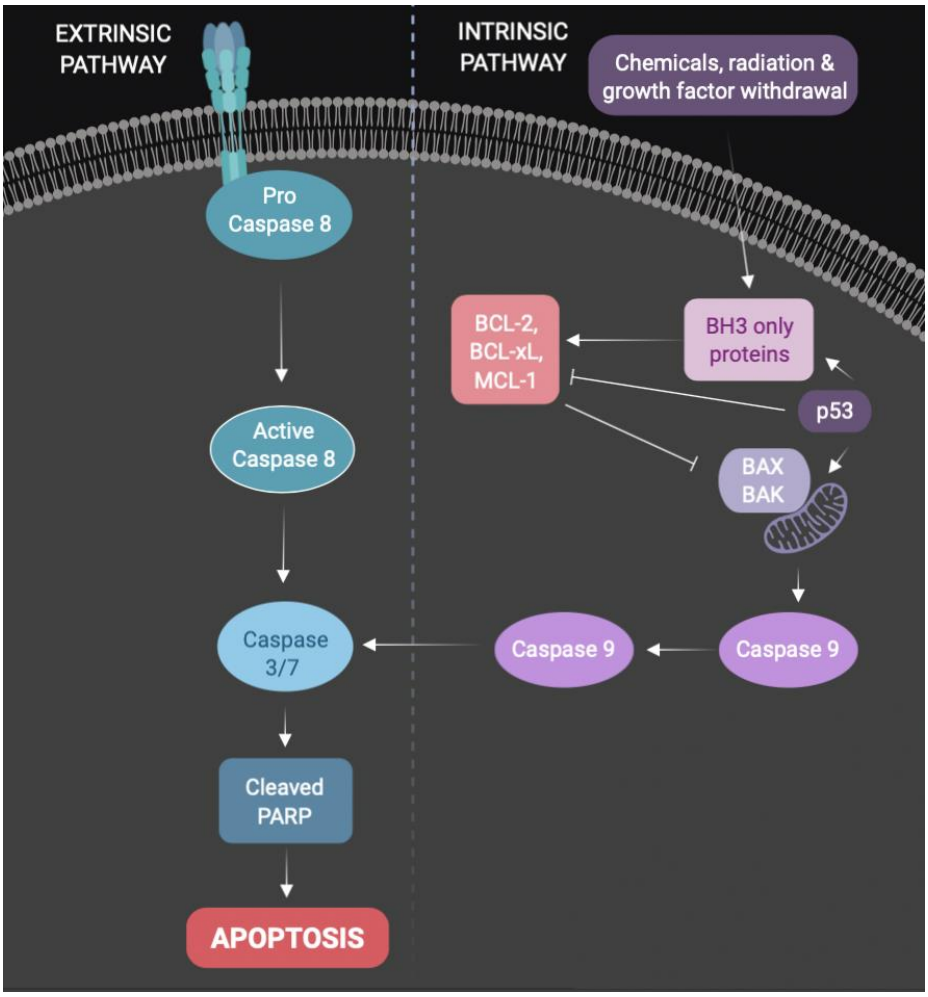


Figure 3. Intrinsic and extrinsic caspase cell signaling pathways. These pathways are involved in regulating apoptosis and represent the different mechanisms of programmed cell death from p53 activation.

1.8 Cancer Cell Enhanced Migration

Matrix metalloproteinases (MMPs) belong to a family of zinc- and calcium-dependent metalloproteinases that are responsible for remodeling the extracellular matrix (ECM) [84,85]. These MMPs are necessary for wound repair and cell growth/development [84]. MMP mRNA expression is typically at low levels, however these levels increase with inflammation, wound healing or in cancer [86]. Enzymes that degrade the ECM are essential for tumor progression,

and can promote cancer cell invasion onto surrounding tissues, entry into blood vessels, and allows cancer cells to metastasize to distant organs [86]. MMP-9 (92 kDa) specifically, plays a key role in these physiological processes and as a result elevated levels of MMP-9 is associated with increased metastatic capabilities in many types of cancer cells, including breast and prostate cancer cells [85].

Different types of cell adhesion are regulated by various adhesion molecules, for example cell to cell adhesions are formed by the E-cadherin complex [87]. For cell invasion to occur single cells must separate from the tumor, this requires a loss of cell-to-cell adhesion and a gain of cell-matrix adhesion properties [87]. The PI3K/Akt and Ras/MAPK pathways can modulate cell migration by increasing downstream MMP-9 production and thus playing a role in the progression of cancer [87].

1.9 Natural Compounds in Cancer Therapy

Over 50% of modern chemotherapeutic agents that are used for cancer treatment have been derived from natural products, either directly from plants or other sources [88,89]. For example the chemotherapeutic treatments paclitaxel and docetaxel, were originally isolated from the bark of the Pacific yew (*Taxus brevifolia*) plant [90]. Many labs, including ours have shown metformin [91,92], a drug derived from the plant *Galega officinalis*, commonly known as French lilac, has anticancer properties. Plant derived extracts with high polyphenolic content such as green tea [93], rosemary extract [94,95], as well as individual polyphenols such as quercetin [96], resveratrol [97–99], oleuropein [100] and others have also shown anti-cancer effects. Rosemary extract (RE) from the plant *Rosmarinus officinalis*, is rich in polyphenols with carnosic acid (CA), rosmarinic acid (RA), and carnosol (COH) found in high concentrations

[95]. RE and RE polyphenols have been reported to have antioxidant and antimicrobial properties [101]. A limited number of studies have found that in various breast and prostate cancer cells rosemary extract can decrease cell viability, inhibit cell proliferation, induce apoptosis, and enhance the effects of various chemotherapeutic drugs [102–105].

1.10 Anti-cancer Effects of Rosemary Extract *(Rosmarinus Officinalis L.)*

1.10.1 Anti-cancer Effects of Rosemary Extract in Breast Cancer: *in vitro* studies

The anti-cancer effects of RE in breast cancer have been studied *in vitro* and it has been shown that in MCF-7 (ER+) and MDA-MB-231 (TN) cell lines using supercritical CO_2 extracted rosemary extract decreased cell viability in a dose dependent manner, with an IC_{50} value of 20.42 µg /mL [102]. In comparison, treatment of breast cancer cell lines with rosemary extract obtained from dried rosemary leaves using the supercritical fluid extraction technique showed that 24 hour supercritical fluid rosemary extract (SFRE) treatment decreased cell viability in five breast cancer cell lines [103]. MCF-7 and UACC-812 were most resistant at higher doses of SFRE, whereas HER-2 positive was most sensitive at low concentrations. It was also found that apoptosis was induced in a dose dependent manner by SFRE in T47D and SK-BR-3 cell lines [103]. However, there was an absence of cleaved PARP1 in the treated breast cancer cell lines, indicating that SFRE may induce apoptosis though a different pathway. SFRE was also found to be more effective on estrogen receptor (ER+) cell lines as it significantly modulated 14 genes in these cell lines compared to 4 genes in ER negative cell lines. It is interesting to note that SFRE downregulated the expression of ESR1, a gene that encodes for ERα [103]. Similarly, SFRE was found to downregulate the HER-2 gene in HER-2 positive breast cancer cell lines [103]. In addition, SFRE enhances the antitumor effect of breast cancer chemotherapy drugs including

paclitaxel, trastuzumab, and tamoxifen. The combination of the extract with these chemotherapeutics showed a significant increase in anti-tumor activity when compared to chemotherapeutic drugs alone [103]. Using the methanol extraction method, rosemary extract was used to treat MDA-MB-453 (ER-), MDA-MB-468 (TN) and MCF7 (ER+) cell lines for 48 hours [106]. It was also found that HER-2 positive cell lines were more sensitive to treatment with RE as the IC_{50} value of HER-2 negative treated cells was 25 µg/mL compared to the IC_{50} value of the HER-2 positive treated cells, which was 9 µg/mL [106]. Furthermore, treatment with various concentrations of rosemary extract (1-100 µg/mL) for 48 hours resulted in an inhibition of MCF-7 breast cancer cell proliferation by 44.86% [107]. Rosemary extract inhibited cell proliferation in a dose-dependent manner, with an IC_{50} value of 16.60 µg/mL [107].

Table 1: Anticancer effects of RE and RE polyphenols *in vitro*: breast cancer.

Cancer Cell	Polyphenol	Dose/ Duration	Findings	Mechanism	Ref
MDA-MB-231 **MCF-7**	RE CA RA	6.25, 12.5, 25, 50, 100 µg/mL RE (48 h) 6.25, 12.5, 25, 50, 100 µM CA and RA (48 h)	CA 50% inhibition of MCF-7 at 24.2 µM concentration. 2-fold higher response in MDA-MB-231.	CA causes arrest of G2/M phase cell cycle affecting cyclin. RA showed proliferative effects. Combination of TNF-α and RA induced apoptosis.	[102]
MDA-MB-231 **MCF-7** **T-47D** **UACC-812** **SK-BR-3** **CCD-812**	Supercritical fluid rosemary extract (SFRE) Containing CA (19.56%) and COH (1.90%)	50 µg/mL (24 h)	Antitumor activity against breast cancer cells. Downregulation of ER- α and HER2 receptors. SFRE enhanced the effect of breast cancer chemotherapy.	SFRE decreased cancer cell viability in a dose-dependent manner. MCF-7 and UACC-812 cells were most resistant at higher doses. SFRE dose dependently induced apoptosis in T-47D and SK-BR-3 cells.	[103]
MDA-MB-231 **MCF-7**	CA (93%)	2.5, 5, 10, 20, 40 µg/mL (24 & 48 h)	CA inhibits cell viability, IC_{50} were 8.5 and 14.02 µg/mL for MDA-MB-321 and MCF-7, respectively.	CA has a chemotactic ability within the generated concentration gradients.	[108]

			Indicates selectivity of CA for estrogen independent cell lines.		
MCF-7 **T47D** **MCF-10A**	CA (98%)	0, 1, 2.5, 5, 10, 20, 40 µM (24 h)	CA showed no significant anti-cancer role in T47D cells. CA alone significantly down-regulated MCF-7 cells in a dose dependent manner. CA and tamoxifen combined therapy significantly suppressed cancer cell proliferation.	Caspase 3/ TRAIL activation. Anti-apoptotic Bcl-xL and Bcl-2 were downregulated. Pro-apoptotic signals Bax and Bad were up-regulated.	[109]
MCF-7	RA		RA inhibited DNA methyltransferases (DNMT) activity (up to 88% inhibition) RA reduced DNMT protein levels.	RA reduced PARG activity.	[110]
MDA-MB-361 **MDA-MB-453**	RA (In an extract with other compounds)	0, 2, 4, 8, 10, 20 µL/mL (72 h)	Significantly decreased cell survival. Induced apoptosis in a dose-dependent manner.		[111]
MCF-7	RA	1, 5, 10, 20 µM/L	Prevent COX-2 activation by AP-1 inducing agents in MCF-7.	RA antagonized ERK1/2 activation.	[112]

				Treatment with RA alone reduced basal recruitment of c-Jun and c-Fos	
MDA-MB-231	COH	25, 50, 100 µM (24 & 48 h)	Decreased cellular viability in a dose dependent manner. COH induced apoptosis through the intrinsic and extrinsic pathways. COH inhibited colony growth.	Induced G2 phase cell cycle block. Cleaved PARP, caspase 3,8,9 increased in concentration in a dose-dependent manner (50-100 µM). Increase in Bax/Bc1-2 ratio in favour of apoptosis induction.	[113]
MDA-231 **HBL-100** **MCF-7** **MDA-361** **MDA-345**	COH	12.5, 25, 50, 100, 200 µM (24, 48, & 72 h)	Viability of all cell lines inhibited by COH in a dose-dependent manner. No effect at concentration lower than 25 µM. COH suppresses the adhesion of cancer cells to fibronectin in a dose-dependent manner. Cells treated with COH + curcumin maintained in	Reduced cyclin D1 expression and induced cleavage of ROCK1 (molecule that had an apoptotic effect on cells).	[114]

			suspension showed greater sensitivity to carnosol.		
MDA-MB-453 **MDA-MD-468** **MCF7**	RE CA RA	0-80 µg/mL	CA inhibits breast cancer cell growth. RA exerts little effects. The presence of HER-2 increased the activity of CA. RE and CA arrest the cell cycle at the G1 phase at low concentrations, and at the G2 phase at high concentrations. CA synergizes with curcumin in triple negative breast cancer cells.	CA induces anti-inflammatory, oxidoreductase, apoptosis, and gluthathione metabolism genes and represses inhibitors of transcription and cell cycle genes. CA and curcumin (as well as digitoxin) inhibit the activity of the purified Na+/K+ ATPase.	[106]
MCF-7	RE	1-100 µg/mL (48 h)	Inhibited MCF-7 cell proliferation by 44.86% in a dose dependent manner (IC_{50} = 16.60 µg/mL).		[107]

1.10.2 Anti-cancer Effects of Rosemary Extract in Breast Cancer: *in vivo* animal studies

The anti-cancer effects of RE in breast cancer were also studied *in vivo*. Intraperitoneal injections of RE and carnosol at 200 mg/kg for 5 days in female rats inhibited the 7,12-Dimethylbenz(a)anthracene (DMBA)-induced mammary adduct formation (44% and 40%, respectively) [115]. Furthermore, administration of CA to mice inoculated with ER positive breast cancer cells resulted in a significant inhibition of tumor growth [109]. Treatment of these mice with carnosic acid and tamoxifen (30 and 10 mg/kg, respectively) as a combined therapy resulted in a greater inhibition of tumor growth in comparison to carnosic acid or tamoxifen monotherapy [109].

Table 2: Anticancer effects of RE and RE polyphenols *in vivo:* breast cancer.

Animal Model	Polyphenol	Dose/ Duration	Findings	Mechanism	Ref
DMBA-induced mammary tumorigenesis (Sprague dawley rats)	RE COH	Intraperitoneal injections 200 mg/kg for 5 days.	Inhibited DMBA-induced mammary adduct formation (44 and 40% with RE and COH treatment, respectively. Both COH and RE effective are inhibitors of initiation state DMBA-induced rat mammary tumorigenesis.	May be due to stimulation of liver detoxification of DMBA.	[115]
MCF-7 xenograft model (Athymic nude mice)	CA	30 mg/kg CA or 10 mg/kg tamoxifen or combined.	Inhibition of tumor growth, both as monotherapies, or as a combined therapy. However, combined therapy showed greater inhibition.		[109]

1.10.3 Anti-cancer Effects of Rosemary Extract in Breast Cancer: *ex vivo* study

Cultured breast tumor explants were obtained from precision-cut breast tumor slices from eleven patients during surgery at the Hospital of Gynecology and Obstetrics from the Mexican Institute of Social Security [116]. The tumor slices were cultured and exposed to varying concentrations of the RE polyphenol rosmarinic acid alone, as well as in combination with the chemotherapeutic agent paclitaxel [116]. It was found that at RE had no effect at concentrations of 60 µg/mL or lower so treatment was increased to 120 µg /mL for 48 hours [116]. At this concentration, the effect of RA as a monotherapy did not decrease cell viability or proliferation. However, RA as a combination therapy with paclitaxel induced a significant 2.34 fold increase in cytotoxicity compared to the untreated control. Therefore, RE and RE polyphenols may have the potential to act as a combined therapy with current chemotherapeutic agents to increase their effect [116].

Table 3: Anticancer effects of RE *ex vivo*: breast cancer.

Tissue	Polyphenol	Dose/ Duration	Findings	Mechanism	Ref
Breast Tumor explants	RA	120 µg /mL (48 h)	Enhanced activity of chemotherapeutics (paclitaxel).		[116]

1.10.4 Anti-cancer Effects of Rosemary Extract in Prostate Cancer: *in vitro* studies

The effect of rosemary extract in prostate cancer cells has been studied *in vitro* and it has been found that in PC-3 and DU145 prostate cancer cells, treatment with supercritical CO_2 extracted rosemary decreased cell viability in a dose-dependent manner [102]. Cells were treated with various concentrations of RE for 48 hours and the results showed that the supercritical fluid extract displayed a greater inhibitory effect compared to the soxhlet extracts in the DU145 prostate cancer cell line [102]. Similarly, it has also been found that 48 hour RE treatment can also inhibit cell viability in 22RV1 and LNCaP prostate cancer cell lines in a dose-dependent manner [117]. IC_{50} values of 13.3 µg/mL and 27 µg/mL were observed for 22RV1 and LNCaP cells, respectively [117]. It was also shown that RE inhibited cell proliferation in 22RV1 and LNCaP cells by 76.5 and 94.6%, respectively [117]. Concentrations as low as 20 µg/mL of RE decreased AR expression and a significant decrease in PSA was shown in LNCaP cells [117].

Table 4: Anticancer effects of RE and RE polyphenols *in vitro*: prostate cancer.

Cancer Cell	Polyphenol	Dose/ Duration	Findings	Mechanism	Ref
PC-3 **DU145**	RE CA RA	6.25, 12.5, 25, 50,100 µg/mL (48 h) 18.8-150 µM CA (48 h) 17.3-138.8 µM RA (48 h)	Decrease in cell viability (IC_{50} = 8.82 µg/mL for RE treatment).		[102]
LNCaP **22RV1**	RE	10-50 µg/mL (24 to 48 h) RE standardized to 40% CA.	Decrease in cell viability and proliferation. Increase in cell cycle arrest and apoptosis.	Increase CHOP, BAX, and cleaved caspase 3. Decrease PSA production and androgen receptor expression.	[117]
PC-3	CA	20-100 µM (0-72 h)	Decreased cell proliferation. Increased apoptosis.	Decreased caspase 8, 9, Bcl-2, Bid, IAP, p-Akt, p-GSK3, and NF-kB. Increased Caspase 3, 7, PARP cleavage, Bax, Cytochrome c, and PP2A.	[118]
PC-3 **LNCaP** **DU145**	CA	10 µM (72 h)	Decreased cell proliferation.	Decreased EpRE/ARE transcription system. Decreased PSA secretion.	[119]

LNCaP **22RV1**	CA	10-100 µM (24 to72 h)	Decreased cell proliferation and viability. Increased apoptosis.	Decreased expression of PSA. Increased cleaved caspase 3 expression.	[120]

1.10.5 Anti-cancer Effects of Rosemary Extract in Prostate Cancer: *in vivo* animal studies

The anti-cancer of RE in prostate cancer using an *in vivo* model showed that treatment of athymic nude mice transplanted with 22RV1 human prostate cancer cells with RE [117] and CA [120] resulted in decreased tumor size. Athymic nude mice were treated with either olive oil or RE dissolved in olive oil for 22 days [117]. Tumor formation was observed at day 14 and by the end of the study period a 46% reduction in tumor size was measured in the RE administered mice when compared to the control [117]. Western blots using lysates from the mouse tissue showed decreased AR and PSA expression in the RE treated group compared to the untreated control [117]. Similarly, athymic nude mice treated with CA dissolved in cotton seed oil for 25 days showed a 53% reduction in tumor growth when compared to the control untreated mice [120]. CA also decreased AR expression, as determined by western blotting control [120].

Although the studies examining the effects of rosemary extract and rosemary extract polyphenols *in vivo* are limited, the data/evidence suggest they may be effective in inhibiting tumor growth as a monotherapy or as a combined therapy with other chemotherapeutic agents.

Table 5: Anticancer effects of RE and RE polyphenols *in vivo*: prostate cancer.

Animal Model	Polyphenol	Dose/ Duration	Findings	Mechanism	Ref
22RV1 xenograft model (Athymic nude mice)	RE	100 mg/kg/day in olive oil, orally (22 days)	Decreased tumor volume (induced apoptosis).	Decreased androgen receptor expression and PSA. Increased CHOP.	[117]
22RV1 xenograft model (Athymic nude mice)	CA	100 mg/mouse dissolved in 100 µL of cottonseed oil by oral gavage (25 days)	53% reduction in tumor growth.		[120]

Chapter 2: Rationale, Objectives and Hypotheses of the Present Study

2.1 Rationale

It has been well established that cancer cells have enhanced proliferative and survival capabilities. These capabilities are the result of mutations in key signaling proteins including ones involved in the PI3K/Akt and Ras/MAPK pathways as discussed in Chapter 1. Many chemotherapeutic agents have been discovered to specifically target these proteins, however due to drug resistance, over time chemo and radio-therapies become ineffective. The discovery of novel chemicals that can be used alone or in combination with existing drugs to treat cancer, especially those that are highly aggressive, is essential to improve patient outcome.

Breast and prostate cancer are the most frequently diagnosed cancers in women and men respectively, in North America [1,2]. TNBC accounts for over 15% of all breast cancer cases and represents an aggressive form of the disease, with limited treatment options, and poor prognosis [30,36]. Due to the absence of estrogen receptor, progesterone receptor, and HER-2 amplification, TNBCs do not benefit from hormone-based therapies [36].

Prostate cancers that do not express PSA and are androgen-independent are highly aggressive and do not respond well to hormone-based therapies [121]. This highlights the necessity to develop new therapeutic strategies to treat breast and prostate cancer.

The Mediterranean diet has long been attributed to preventing or delaying the onset of cardiovascular disease, diabetes, and various cancers. One component of this diet that is commonly used is the herb rosemary. Rosemary extract from the plant *Rosmarinus officinalis*, is rich in polyphenols with carnosic acid, rosmarinic acid, and carnosol found in high

concentrations [95]. RE and RE polyphenols have been reported to have antioxidant and antimicrobial properties [101]. Rosemary extract has also shown to exert anti-cancer effects both *in vitro* and *in vivo* across various types of cancers [95]. Limited data exists regarding the effects of rosemary extract in TNBC and prostate cancer, and little is known about the underlying signaling mechanisms involved in its anti-proliferative properties.

Historically, many plants and food components have been the basis for isolation of compounds that are used in the treatment of various illnesses. Acetylsalicylic acid (Aspirin), commonly used to treat minor pain and reduce inflammation, was isolated from the willow tree, and morphine, used to treat pain, is an opioid isolated from the opium poppy. Recently, plant derived extracts with high polyphenolic content such as green tea [93], rosemary extract [95], as well as individual polyphenols such as quercetin [96], resveratrol [97,98], oleuropein [100] and others have also shown anti-cancer effects. Thus, further investigation of rosemary may lead to the discovery of chemicals with potent anticancer properties.

2.2 Objectives

The objectives of the present study were to:

a) Examine the effects of rosemary extract on proliferation, survival, and migration in MDA-MB-231 triple negative breast cancer and PC-3 prostate cancer cells.

b) Investigate the effects of RE on key signaling proteins involved in the pathways that regulate cell proliferation, survival/apoptosis, and migration.

2.3 Hypotheses

In the present study it was hypothesized that:

a) Rosemary extract inhibits cell proliferation, survival, and migration of MDA-MB-231 triple negative breast cancer and PC-3 prostate cancer cells in a dose-dependent manner.

b) Rosemary extract modulates the signaling cascades involved in regulating cell proliferation and survival/apoptosis in MDA-MB-231 breast and PC-3 prostate cancer cells.

    i. RE inhibits the PI3K/Akt and Ras/MAPK pathways, as well as induce apoptosis by increasing PARP cleavage.

Chapter 3: Methodology

3.1 Cell Lines

Cells in culture were used to investigate the anticancer effects of rosemary extract and its main polyphenolic constituents. These cells resemble the primary tumor population but offer a homogenous population of cells with unlimited replicative capacity. The use of cultured cells to investigate the physiology and biochemistry of cells and intracellular signaling pathways is well-established. The cell lines to be used in this study are the triple negative breast cancer cells (MDA-MB-231) and androgen insensitive prostate cancer cells (PC-3). These cells will be used for experiments *in vitro* and detailed cellular mechanisms/pathways elucidated.

The **MDA-MB-231** cell line was isolated from pleural effusions of a 51-year-old female Caucasian with breast cancer [122]. This cell line does not express estrogen receptors and therefore has been widely used to examine non-hormone sensitive breast cancer.

The **PC-3** cell line was derived from a 62-year-old Caucasian male from the bone metastasis of a grade IV prostatic adenocarcinoma [123]. These cells do not express the androgen receptor and therefore are a widely used *in vitro* model of hormone insensitive prostate adenocarcinoma.

Table 6: Breast/prostate cancer cell line properties.

Cell Line	Histology	Mutation Status/ Hormone Responsiveness
MDA-MB-231	Breast adenocarcinoma	p53 null KRAS mutation Estrogen/ progesterone receptor negative Human epidermal growth factor receptor-2 negative
PC-3	Prostate adenocarcinoma	p53 null PTEN null Androgen insensitivity Prostate specific antigen negative

3.2 Materials

The MDA-MB-231 (adenocarcinoma) and PC-3 (adenocarcinoma) cancer cells were obtained from American Type Culture Collection (ATCC) (Virginia, USA). The Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum (FBS), 0.25% trypsin and the antibiotic-antimycotic solution were purchased from GIBCO Life Technologies (Burlington, ON, Canada). Akt (#9272) (1:1000 dilution), p-Akt (Ser473) (#9271) (1:1000 dilution), mTOR (#2972) (1:1000 dilution), p-mTOR (#2971) (1:1000 dilution), PARP (#9542) (1:1000 dilution), β-actin (#8457) (1:1000 dilution), as well as secondary anti-rabbit IgG HRP-linked antibodies (#7074) (1:2000 dilution) were from Cell Signaling Technology via New England Biolabs (Mississauga, ON, Canada). Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), Paclitaxel, and Metformin were from Sigma (Oakville, ON, Canada). Clarity Western enhanced chemiluminescence substrate (ECL), 30% acrylamide/bis solution 37 (5:1), ammonium persulfate (APS), polyvinylidene difluoride (PVDF) membranes and reagents for electrophoresis were purchased from Bio-Rad (Hercules, CA, USA).

3.3 Buffers and Solutions

The buffers and solutions that will be required for each experiment and their compositions are provided below:

Cell Lysis

Phosphate Buffered Saline (PBS) Solution- Wash Buffer

137 mM NaCl; 2.7 mM KCl; 1.5 mM KH_2PO_4; 8.1 mM Na_2HPO_4; 0.68 mM $CaCl_2$; 0.49 mM $MgCl_2$; Add distilled water to achieve final volume, adjust pH to 7.4 and sterilize (autoclave) before use.

Cell Lysis Buffer

20 mM Tris-HCl solution (pH 7.5); 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 2.5 mM $Na_4P_2O_7$; 1mM glycerol 2-phosphate; 1 mM Na_3VO_4; 1 μg/mL leupeptin; add 1 mM PMSF before use and chill on ice.

4X SDS Sample Buffer

240 mM Tris-HCl solution (pH 6.8); 8% w/v SDS; 40% glycerol, 0.04% bromophenol blue; deionized water; add 5% β-mercaptoethanol before use.

Western blotting solutions

1.5 M Tris-HCl (pH 8.8)

27.23g Tris base (18.15 g/100 mL); 80 mL deionized water; adjust pH to 8.8 with 6 N HCl and bring total volume to 150 mL with deionized water

0.5 M Tris-HCl (pH 6.8)

6 g Tris base (6 g/100 mL); 60 mL deionized water; adjust pH to 6.8 with 6 N HCl and bring total volume to 100 mL with deionized water.

Resolving Gel Buffer (10%) – for 4 1.5 mm gels

12.3 mL deionized water; 9.9 mL 30% acrylamide/Bis solution; 7.5 mL 1.5 M Tris-HCl (pH 8.8); 0.3 mL 10% w/v SDS. Right before pouring gel add 150 μL 10% APS (0.01 g into 100 μL deionized water) made fresh daily and 30 μL TEMED and swirl.

Stacking Gel Buffer (4%) – for 4 1.5 mm gels

18.3 mL deionized water; 3.9 mL 30% acrylamide/Bis solution; 7.5 mL 0.5M Tris-HCl (pH 6.8); 0.3 mL 10% w/v SDS. Right before pouring gel add 150 μL 10% APS (0.01 g into 100 μL deionized water) made fresh daily and 30 μL TEMED and swirl.

10x Tris-Buffered Saline (TBS)

24.2 g Tris base; 80 g NaCl; fill to total volume of 1 L with deionized water; adjust pH to 7.6 with HCl. Use as 1x TBS/T (50 mL 10x TBS into 450 mL deionized water; then add 500 μL Tween).

10x Electrode Running Buffer

15.15 g Tris base; 72 g glycine; 5 g SDS; dissolve and bring volume to 500 mL with deionized water. Do not adjust pH. Before use dilute to 1x (50 mL 10x stock into 450 mL deionized water).

Wash Buffer

1x TBS and 0.1% Tween-20.

Transfer Buffer

25 mM Tris base (3.03 g); 0.2 M glycine (15.01 g); 20% methanol (200 mL/800 mL deionized water); adjust final volume to 1 L.

Blocking Buffer

15 mL 10x TBS; 135 mL deionized water; 7.5 g nonfat dry milk (5% w/v); 0.15 mL Tween; make fresh each time.

Primary Antibody Dilution Buffer

2 mL 10x TBS; 18 mL deionized water; 1.0 g BSA; 20 µL Tween (add after first 3 ingredients are mixed); add 10 µL primary antibody before use.

Secondary Antibody Buffer

10 mL blocking buffer; 5 µL secondary antibody; make fresh each time.

Crystal violet assay solutions

Crystal Violet Stain

0.5% w/v crystal violet stain in 25% v/v methanol; keep solution covered from light

Solubilizer Solution

0.05 M NaH_2PO_4 (MW 119.98 g/mol) in 50% v/v ethanol; keep solution covered from light

Wound healing assay solution

Mitomycin-C

1 µg/mL mitomycin-C prepared in RPMI or DMEM media.

3.4 Cell Culture

MDA-MB-231 are epithelial breast adenocarcinoma cells. The MDA-MB-231 breast cancer cells were cultured in DMEM media supplemented with 1% (v/v) antibiotic- antimycotic (100 µg /mL penicillin, 100 µg/mL streptomycin and 250 ng/mL amphotericin) and 10% (v/v) FBS. PC-3 are epithelial prostate adenocarcinoma cells. The PC-3 prostate cancer cells were cultured in RPMI 1640 media supplemented with 1% (v/v) antibiotic- antimycotic (100 µg /mL penicillin, 100 µg/mL streptomycin and 250 ng/mL amphotericin) and 10% (v/v) FBS. Media was changed every 2-3 days and cells were trypsinized with 0.25% trypsin EDTA when 70-80% confluency was achieved. Cells were grown in an incubator at 37℃ and 5.0% carbon dioxide (CO_2)- 95% air. Cell handling was performed in Forma Class II, A2 Biological Safety Cabinet.

3.5 Rosemary Extract Preparation

Whole dried rosemary (*Rosmarinus officinalis L.*) leaves (purchased from Compliments/Sobeys, Mississauga, ON, Canada) were used, and the rosemary extract was prepared as previously reported [124]. Briefly, dried rosemary leaves were ground and steeped

overnight (16 hours) in dichloromethane: methanol (1:1) followed by filtration the next day. After filtering, the liquid solvent was set aside while the leaves were boiled in methanol for 30 minutes. The liquid solvent obtained after boiling was combined with the filtered liquid solvent. The combined solvent was removed from the final extract by rotary evaporation and the green powder was collected and stored at -20℃, protected from light. Aliquots were prepared in dimethyl sulfoxide (DMSO) to yield a final concentration of 100 mg/mL.

3.6 Crystal Violet Assay

The crystal violet assay was used to assess cell proliferation/ growth. The crystal violet binds and stains the DNA of the cells, thus allowing for the measurement of the cells ability to divide after exposure to a specific treatment. Cells were seeded (1000; counted using hemocytometer) in 100 µL supplemented DMEM or RPMI 1640 media in triplicate wells in 96-well plates and allowed to adhere overnight. The following day, treatments were added with an extra 100 µL media, with a final volume of 200 µL, and was incubated for 72 hours. After the 72 hour time course, the media was removed, and the cells were fixed with 10% formalin (100 µL per well). The wells were then rinsed using sterile 1X phosphate buffered saline (PBS) and stained using 0.5% crystal violet stain in 25% v/v methanol (50 µL/ well). The excess stain was removed by submerging the plates three times in tap water, and the plates were then allowed to dry overnight. The next day solubilizer solution composed of 0.05 M NaH_2PO_4 in 50% v/v ethanol was added (100 µL/well) and absorbance was read at 570 nm using the KC4 microplate reader.

3.7 Clonogenic Survival

The clonogenic assay was used to assess the colony forming abilities of the cells, which is an enhanced characteristic of cancer cells, enabling the formation of tumors and allows the cancer to become more aggressive. Cells were seeded (1000; counted using hemocytometer) in duplicates in 2 mL supplemented in 6-well plates and allowed to adhere for 24 hours. The following day, the media was removed, and treatments were added (2 mL per well) and the cells were maintained in the incubator for seven days. At the end of the time course, the media was aspirated, and cells were washed twice with sterile 1X phosphate buffered saline (PBS) and then stained with 0.05% w/v methylene blue in distilled water. Cells were stained for 15 minutes, the excess stain was recovered, and the cells were left on the counter to dry overnight. The next day, colonies greater than 50 cells were counted under the microscope.

3.8 Wound Healing Assay

The wound healing assay was used to assess the migration abilities of the cells. The migrating ability of the cells may indicate the capabilities of the cells to metastasize. Cells were seeded at a density of 2.5 x 10_5 cells/mL into a 6-well plate and the media was replaced every 48 hours until the cells reached 90-100% confluency. When confluency was reached the cells were incubated with mitomycin-C (MMC) (1 μg/mL) for 1 hour to prevent cell proliferation. After the incubation period, a vertical line is drawn in the centre of each well using a 100 μL pipette tip. The wells were drained of media and washed twice with PBS to get rid of floating cells. Each well was then treated with rosemary extract and paclitaxel, respectively. Before taking photographs, horizontal lines were drawn underneath the well plates to be used as a reference for future time points. Photos were taken at 0 and 40-hour time points. Wound closure percentage

was calculated using the equation $\frac{\text{0hr Area—40 hr Area}}{\text{0 Hr Area}}$ x 100%. The area of each wound was measured using the ImageJ software.

3.9 Cell Lysis

Cells were seeded in triplicate in 6-well plates and maintained in the incubator until 90-100% confluency was achieved. MDA-MB-231 cells were serum deprived prior to being exposed to treatments, all cells were serum deprived for 48 hours total. Cells were treated with 50 µg/mL of rosemary extract for the indicated time-period in 10% FBS containing media. Following treatment, cells lysis was performed using cell lysis buffer which contained phenlymethylsulfonyl fluoride (PMSF) protease inhibitor (150 µL/well) and scraped with a plastic scraper. The lysates were then pipetted into 1.5 mL Eppendorf tubes and 50 µL was removed to be used for the protein assay. Lysates were dyed using 4x SDS sample buffer by adding 1:4 ratio of dye to lysate. Samples were then boiled for 5 minutes and stored in -20 ℃.

3.10 Protein Assay

The protein assay was performed to determine the correct loading volume of each sample to be used in western blotting. Cell lysate samples were pipetted in triplicates into a 96-well plate (10 µL/well) as well as 200 µL/well of Bio-Rad protein assay dye reagent (1:4 dye reagent: distilled water). Samples were incubated for 5-10 minutes and absorbance was measured at 595 nm using the KC4 microplate reader. The samples absorbance as compared to the standard absorbance in order to calculate the final protein concentrations in the samples.

3.11 Western Blotting

Western blotting is a technique used to examine the inhibition or activation of specific signaling cascades by measuring the total and phosphorylated levels of proteins of interest, such as Akt or mTOR. Cell lysate samples containing 20 µg of protein, determined using the Bradford assay [125], were loaded onto 10% polyacrylamide gel and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then transferred onto polyvinylidene difluoride (PVDF), membrane which was exposed to blocking buffer (5% (w/v) dry milk in Tris-buffered saline) for 1 hour and incubated with the primary antibody overnight at 4°C. The following day the membrane was incubated with horse radish peroxidase (HRP)-linked IgG anti-rabbit secondary antibody for 1 hour at room temperature. Enhanced chemiluminescence (ECL), the Bio-Rad Clarity Western solution, was used to detect the bands corresponding to the proteins of interest. Densitometric analysis was performed using ImageJ software. The data (arbitrary densitometric units) were corrected to β-actin levels and expressed as a percentage of untreated control cells.

3.12 Statistical Analysis

The results are the mean ± standard error mean (SEM) of the indicated number of independent experiments. Analysis of Variance (ANOVA) followed by Bonferroni's post-hoc test was used to determine the significance of the differences between groups. Statistical significance was assumed at $p<0.05$. Statistical tests were performed using GraphPad Prism 8 software.

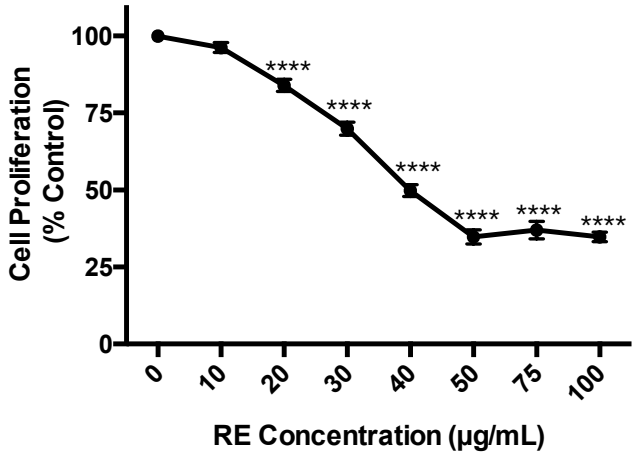
Chapter 4: Results

4.1 Biological effects of rosemary extract (RE) in triple negative breast cancer cells

4.1.1 Effects of RE on cell proliferation in MDA-MB-231 breast cancer cells

The triple negative MDA-MB-231 breast cancer cells were exposed to 10, 20, 30, 40, 50, 75, or 100 µg/mL RE for 72 hours and cell proliferation was assessed using the crystal violet assay. Treatment with RE resulted in a dose–dependent inhibition of cell proliferation. A significant inhibition (83.95 ±1.96 % of control, $p<0.0001$) was seen with 20 µg/mL RE while maximum inhibition (34.79 ±2.32 % of control, $p<0.0001$) was seen with 50 µg/mL RE (Figure 4A and B). Higher RE concentrations (75 and 100 µg/mL) did not result in any greater inhibition of cell proliferation (Figure 4A). The IC_{50} of RE for cell proliferation was 28.86 µg/mL. Previous studies by our group [92,126] and others [127,128] have shown anticancer effects of metformin, a drug used to treat hyperglycemia /type 2 diabetes mellitus [129], and therefore, metformin was used in the present study to compare its effects with the effects of RE. In addition, paclitaxel, derived from *Taxus brevifolia* is an established medication used clinically in the treatment of triple negative breast cancer [130] and we used it in the present study to compare the effects of RE to the effects of paclitaxel. Treatment of the cells with 5 mM metformin did not result in any significant inhibition of cell proliferation (100.5 ±2.10% of control) (Figure 4B), while treatment with 10 nM of paclitaxel showed significant inhibition of cell proliferation (72.31 ±10.77% of control, $p<0.01$) (Figure 4B). Importantly, the inhibition of cell proliferation seen with 50 µg/mL of rosemary extract (34.79 ±2.32 % of control, $p<0.0001$) was greater than that seen with 10nM of paclitaxel (72.31 ±10.77% of control, $p<0.01$).

(A)



(B)

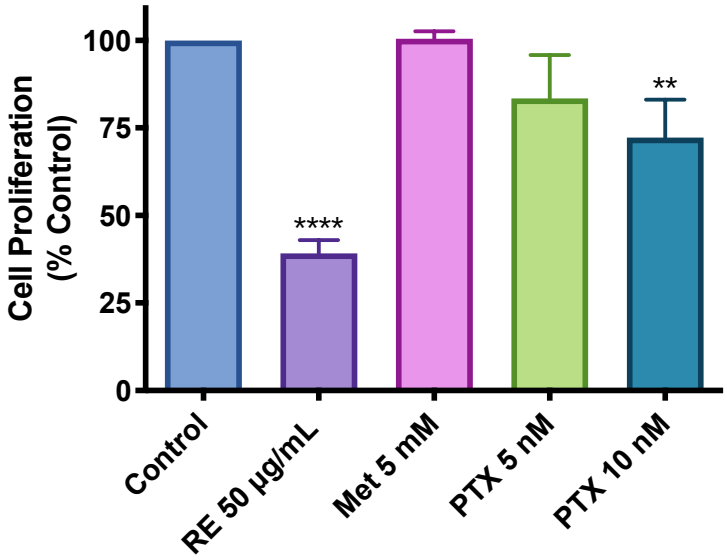
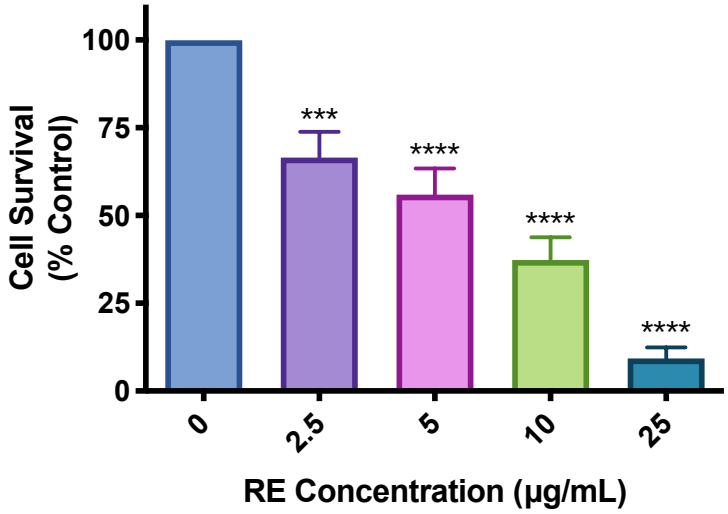


Figure 4. Effect of rosemary extract on MDA-MB-231 breast cancer cell proliferation. MDA-MB-231 cells were treated without (control) or with the indicated concentrations of rosemary extract (RE) (A, B), metformin (Met) (B), or paclitaxel (PTX) (B) for 72h followed by fixing and staining with 0.5% crystal violet. The stain was solubilized, and absorbance was read at 570 nm. Data are the mean ± SEM of 6 independent experiments. **p<0.01, ****p<0.0001.

4.1.2 Effects of RE on cell survival in MDA-MB-231 breast cancer cells

The ability of cancer cells to survive and form colonies was assessed using the clonogenic survival assay. Exposure of MDA-MB-231 cells to 2.5, 5, 10, or 25 µg/mL of RE resulted in a concentration-dependent inhibition of survival (Figure 5A). The lowest concentration of RE that resulted in a significant inhibition of cell survival was 2.5 µg/mL (66.47 ±7.39% of control, $p<0.001$). The greatest inhibition (9.33 ±3.09% of control, $p<0.0001$) of cell survival was seen at 25 µg/mL RE (Figure 5B). Based on our data we calculated the RE concentration for the half maximal inhibition (IC_{50}) of cell survival was 4.82 µg/mL. Exposure of the cells to 5mM of metformin was found to significantly inhibit MDA-MB-231 cell survival (63.36 ±6.01% of control, $p<0.0001$) (Figure 5B). In addition, treatment with 2 nM of paclitaxel resulted in a significant inhibition of cell survival (47.28 ±3.13% of control $p<0.0001$). It should be noted that the inhibition of MDA-MB-231 cell survival seen with 25 µg/mL of RE (9.33 ±3.09% of control $p<0.0001$) was much greater than that seen with metformin (63.36 ±6.01% of control, $p<0.0001$) or paclitaxel (47.28 ±3.13% of control, $p<0.0001$) treatment.

(A)



(B)

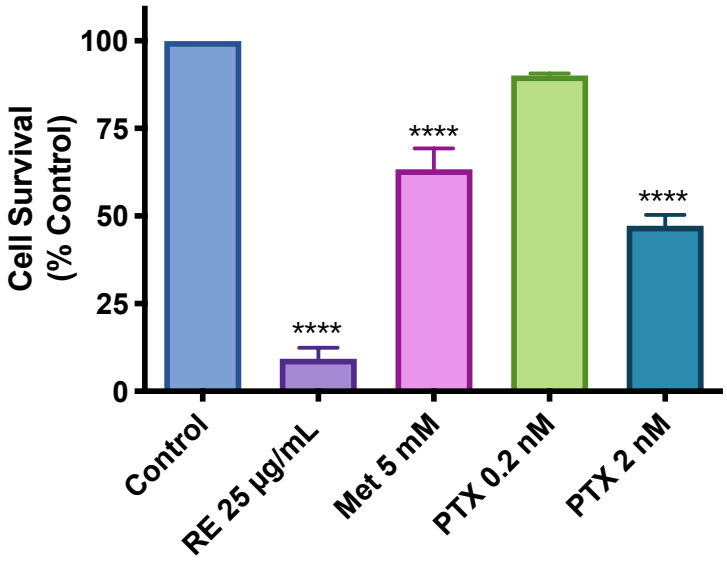
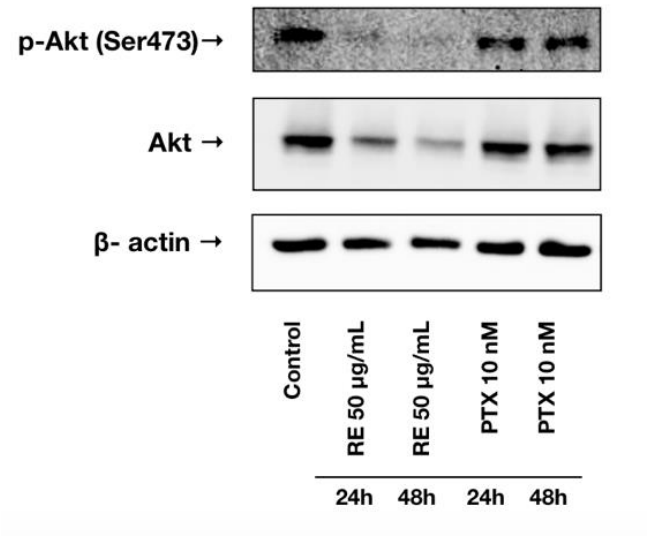


Figure 5. Effect of rosemary extract on MDA-MB-231 breast cancer cell survival. MDA-MB-231 cells were seeded (1000 cells/well) in a 6-well plate and exposed to the indicated concentrations of rosemary extract (RE) (A, B), metformin (Met) (B), or paclitaxel (PTX) (B) for 7 days followed by fixing and staining with 0.05% methylene blue. Colonies of more than 50 cells were counted. Data are the mean ± SEM of 6 independent experiments. ***p<0.001, ****p<0.0001.

4.1.3 Effects of RE on Akt signaling in MDA-MB-231 breast cancer cells

Next, we examined the effects of RE treatment on the activation of the pro-survival kinase Akt and measured the levels of Akt phosphorylation on the serine 473 residue, an established indicator of Akt activity [131]. Treatment of MDA-MB-231 breast cancer cells with rosemary extract (50 µg/mL) for 24 hours (25.62 ±2.56% of control, $p<0.0001$) and 48 hours (11.05 ±0.62% of control, $p<0.0001$) resulted in a significant decrease in Akt phosphorylation/activation (Figure 6A and 6B). The total Akt levels were also significantly inhibited by RE treatment for 24 (36.18 ±5.75% of control, $p<0.0001$) and 48 (18.89 ±5.99% of control, $p<0.0001$) hour treatment. Treatment of MDA-MB-231 cells with paclitaxel for 24 hours also showed a significant of inhibition of Akt phosphorylation/activation (50.06 ±14.25% of control, $p<0.01$). Total Akt levels were also decreased with 24 hour RE treatment in MDA-MB-231 cells (82.78 ±4.02% of control, $p<0.01$). Similarly, treatment of MDA-MB-231 cells with paclitaxel for 48 hours also resulted in a significant inhibition of Akt phosphorylation/activation (45.59 ±16.47% of control, $p<0.001$) and total Akt levels (80.05 ±8.842% of control, $p<0.001$) (Figure 6B).

(A)



(B)

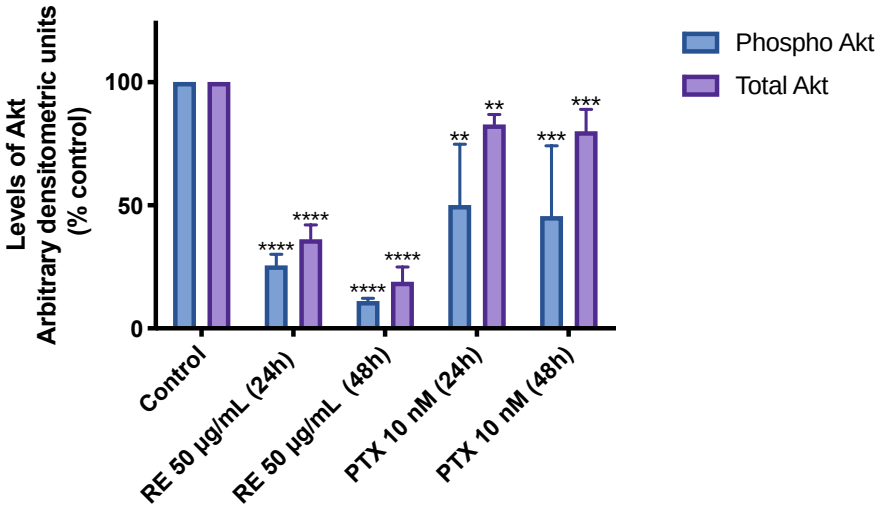
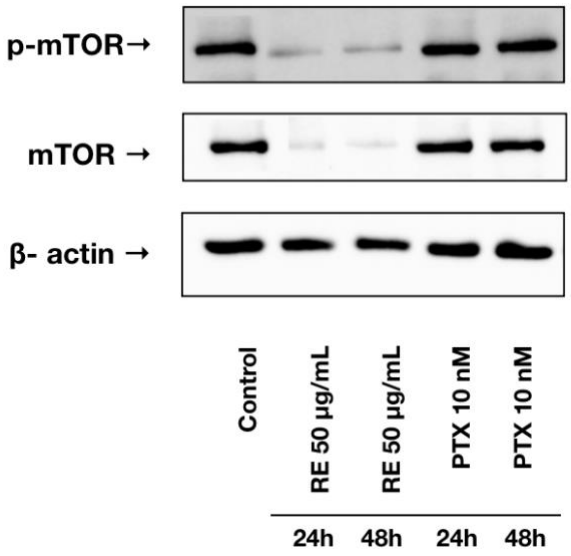


Figure 6. Effect of rosemary extract on Akt signaling in MDA-MB-231 breast cancer cells. Cell lysates were prepared from MDA-MB-231 breast cancer cells treated without (control) or with either 50 µg/mL rosemary extract (RE) or 10 nM paclitaxel for 24 or 48 hours. Cell lysates (20 µg) were immunoblotted using specific antibodies against Akt, phosphorylated/activated Akt, or β-actin. A representative immunoblot is shown (A). The densitometry of the bands were corrected to β-actin levels and expressed in arbitrary densitometry units as % of control. The data are the mean ± SEM of 3 independent experiments. **p<0.01, ***p<0.001, ****p<0.0001.

4.1.4 Effects of RE on mTOR signaling in MDA-MB-231 breast cancer cells

We also examined mTOR phosphorylation/activation levels. Treatment of MDA-MB-231 cells with RE for 24 hours resulted in a significant decrease in mTOR phosphorylation (20.78 ±3.90% of control, $p<0.0001$). Additionally, 48 hour RE treatment also resulted in a significant inhibition of mTOR phosphorylation/activation (12.49 ±5.04% of control, $p<0.0001$) (Figure 7A and 7B). Treatment with RE for 24 hours significantly inhibited total-mTOR expression (8.11 ±1.32% of control, $p<0.0001$). Similarly, treatment with RE for 48 hours also inhibited total-mTOR levels (5.34 ±3.28% of control, $p<0.0001$). Exposing MDA-MB-231 cells to paclitaxel for 24 hours did not result in a statistically significant inhibition of mTOR phosphorylation/activation (101.35 ±8.62% of control, $p>0.05$). A similar trend was also seen for 48 hour RE treated MDA-MB-231 cells (97.58 ±12.53% of control, $p>0.05$) (Figure 7B). Importantly, RE treatment was able to modulate mTOR expression in these cells at both treatment durations.

(A)



(B)

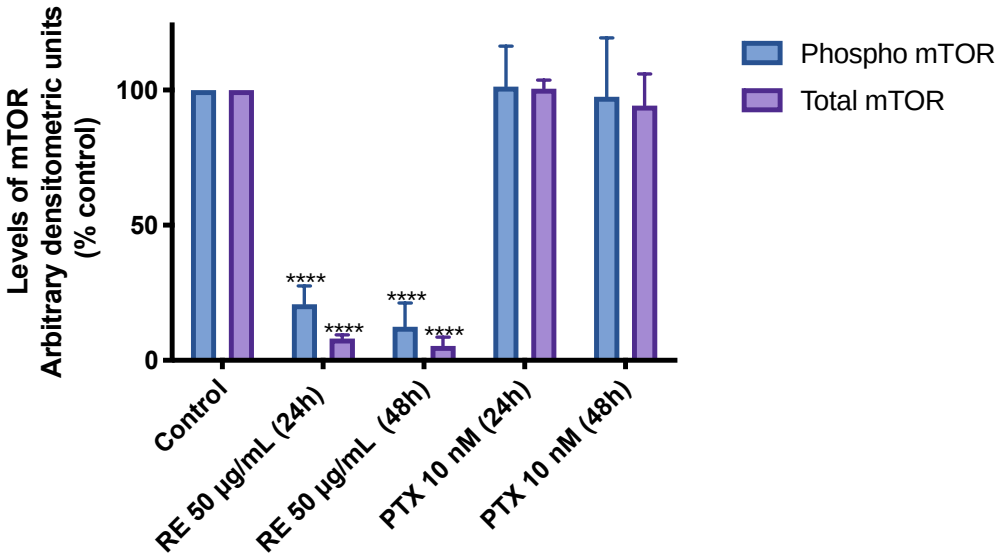
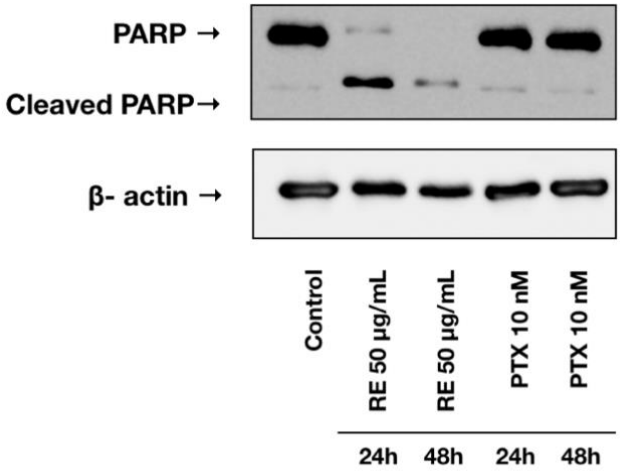


Figure 7. Effect of rosemary extract on mTOR signaling in MDA-MB-231 breast cancer cells. Cell lysates were prepared from MDA-MB-231 cells treated without (control) or with either 50 µg/mL rosemary extract (RE) or 10 nM paclitaxel (PTX) for 24 and 48 hours. Cell lysates (20 µg) were immunoblotted using specific antibodies against mTOR, phosphorylated/activated mTOR or β-actin. A representative immunoblot is shown (A). The densitometry of the bands were corrected to β-actin levels and expressed in arbitrary densitometry units as % of control (B). The data are the mean ± SEM of 3 independent experiments. ****$p<0.0001$.

4.1.5 Effects of RE on apoptosis in MDA-MB-231 breast cancer cells

Next, we examined the effect of RE on cell apoptosis by measuring the levels of cleaved PARP, since it is an established indicator of apoptosis [80]. Exposing the MDA-MB-231 cells to rosemary extract (50 µg/mL) for 24 and 48 hours resulted in a significant increase in the cleaved PARP/PARP ratio (41.4- and 17.5-fold increase, respectively) relative to the control indicating enhanced apoptosis (Figure 8A and 8B). Treatment of MDA-MB-231 cells with 10 nM paclitaxel for 24 or 48 hours did not result in any changes to the level of cleaved PARP (Figure 8B).

(A)



(B)

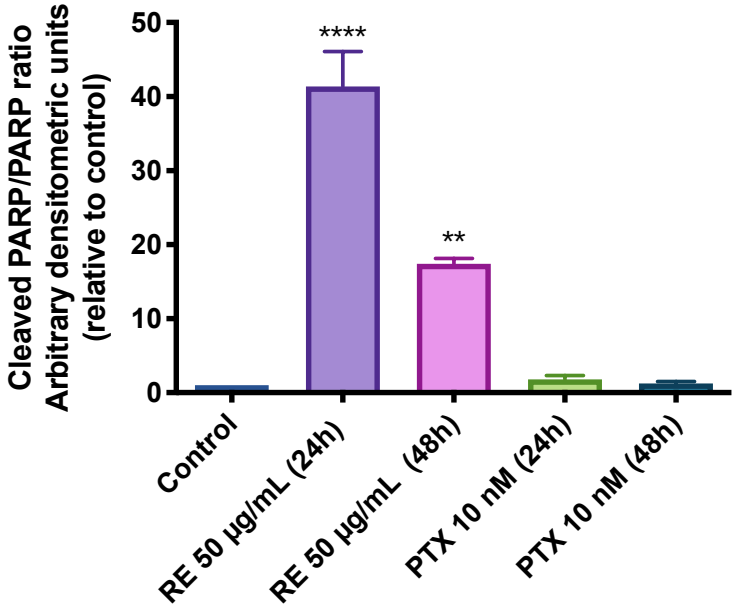


Figure 8. Effect of rosemary extract on MDA-MB-231 breast cancer cell apoptosis. Cell lysates were prepared from MDA-MB-231 TN breast cancer cells treated without (control) or with either 50 µg/mL RE or 10 nM paclitaxel for 24 or 48 hours. Cell lysates (20 µg) were immunoblotted using specific antibodies against PARP or β-actin. A representative immunoblot is shown (A). The densitometry of the bands were corrected to β-actin levels and expressed as a fold increase of arbitrary densitometry units as % of control (B). The data are the mean ± SEM of 3 independent experiments. **p<0.01, ****p<0.0001.

We routinely examined microscopically the effect of our treatments on cell morphology. Figure 9 shows a representative image of control untreated, RE treated, and PTX treated MDA-MB-231 cells. No changes in cell morphology were observed with any of the treatments. It should be noted that the same number of cells were seeded in all wells. As it can be seen from Figure 9, treatment with 50 µg/mL of RE for 24 and 48 hours resulted in a substantial reduction of cell density relative to the control untreated groups. Although treatment with PTX for 24 and 48 hours showed a reduction in cell density, it was not as great as the inhbition seen with RE.

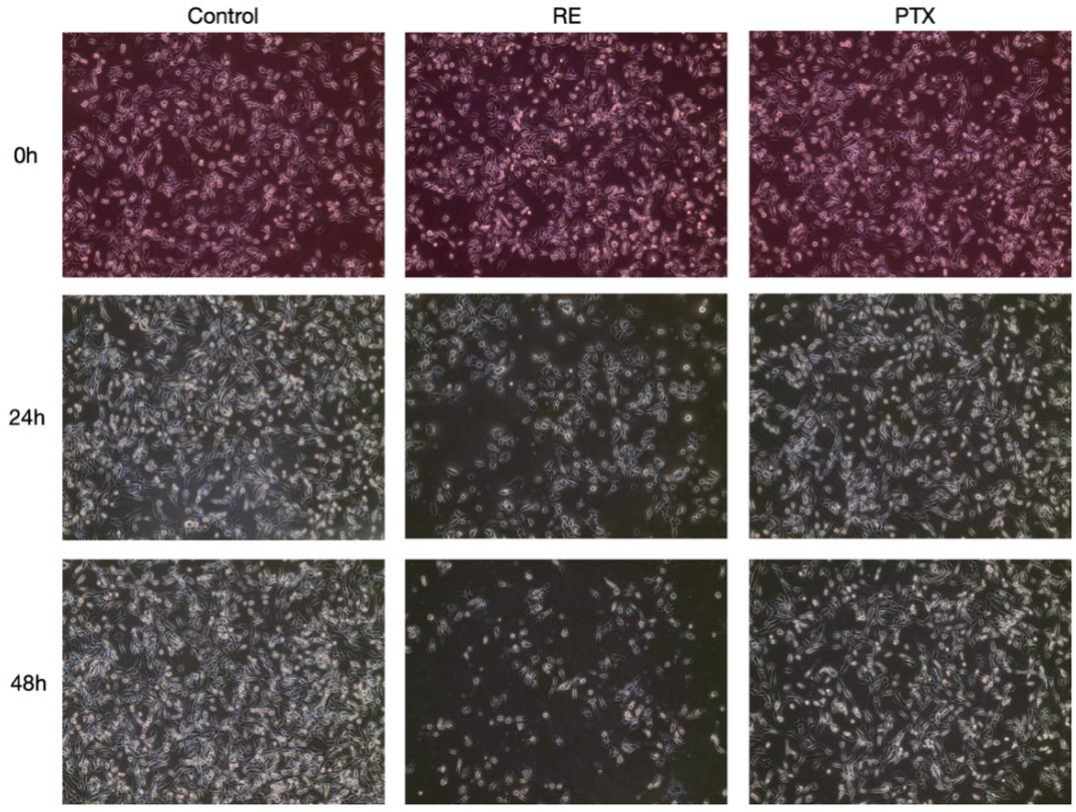
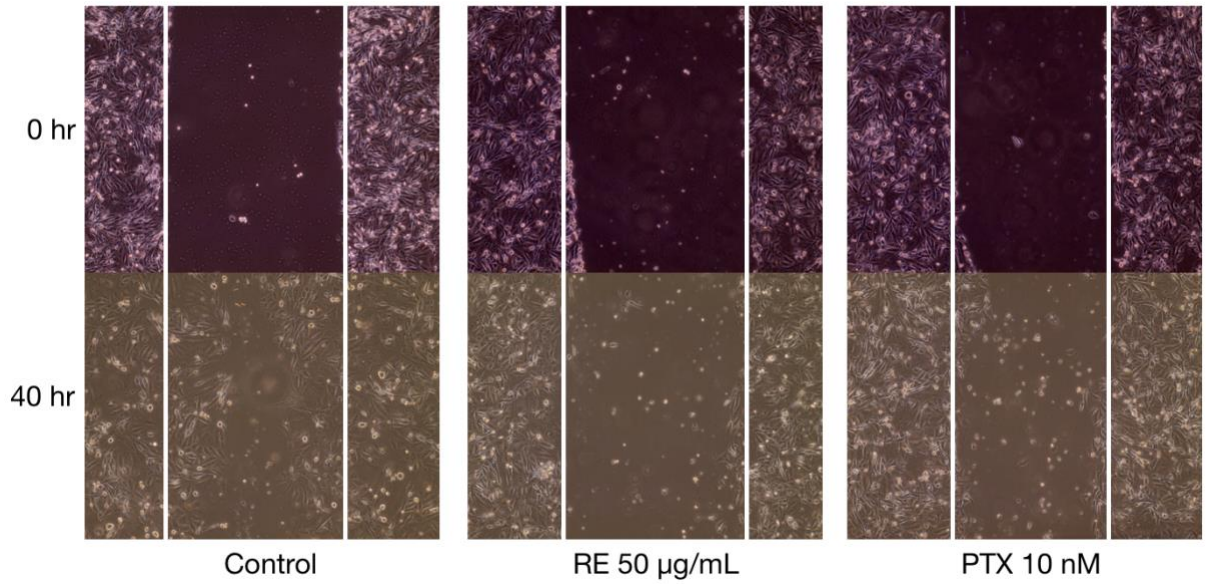


Figure 9. Effect of rosemary extract and paclitaxel on MDA-MB-231 cell morphology. Cells were treated without (control) or with RE (50 µg/mL) or PTX (10 nM) for 24 or 48 hours. Photographs were taken immediately after treatment (0 hr) and after the inidctaed time points using an EVOS XL Core Cell Imaging System by Life Technologies (10X magnification).

4.1.6 Effects of RE on MDA-MB-231 breast cancer cell migration

The ability of cancer cells to migrate was assessed using the wound healing assay [132–134]. MDA-MB-231 cells were exposed to 1 µg/mL of mitomycin-C (MMC) for 1 hour to inhibit cell proliferation and were then treated without (control) or with either 50 µg/mL rosemary extract or 10 nM paclitaxel for 40 hours, just before complete wound closure. Treatment with rosemary extract was shown to significantly inhibit wound closure (65.15 ±0.97% of control, $p<0.001$) indicating properties against cell migration (Figure 10B). A significant inhibition of cell migration was also seen when treating the cells with 10 nM paclitaxel (78.44 ±5.60%, $p<0.01$) (Figure 10A and 10B).

(A)



(B)

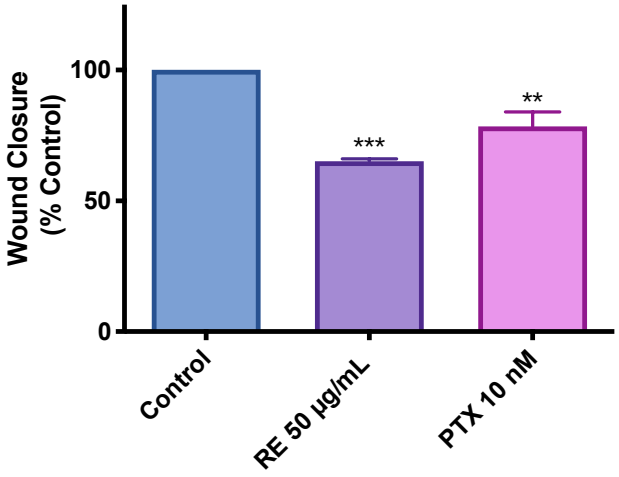


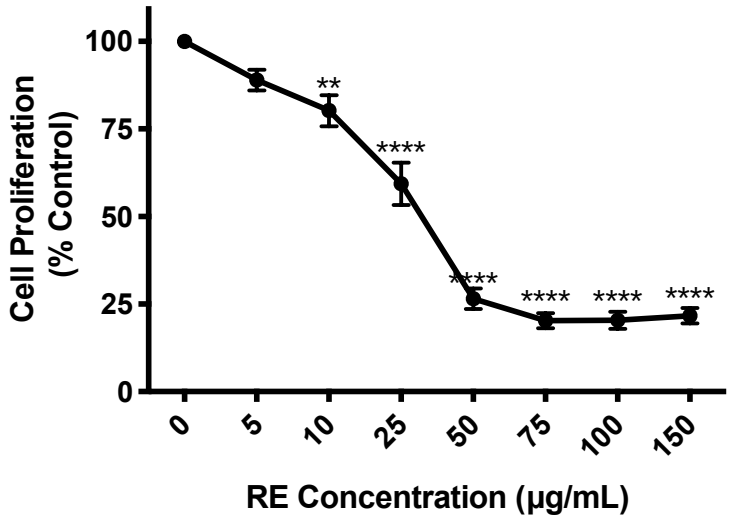
Figure 10. Effect of rosemary extract on MDA-MB-231 breast cancer cell migration. MDA-MB-231 cells were exposed to 1 µg/mL of mitomycin-C for one hour, followed by treated without or with 50 µg/mL rosemary extract (RE) or 10 nM paclitaxel (PTX) for 40 hrs. Representative images of wound healing are shown immediately after being scratched with a 200 µL pipette tip (0 hr) and after 40 hours of treatment (A). Wound closure was calculated as indicated in the methods and expressed as a percent of control untreated cells (B). The data are the mean ± SEM of 3 independent experiments. **p<0.01, ***p<0.001.

4.2 Biological effects of rosemary extract (RE) in prostate cancer cells

4.2.1 Effects of RE on cell proliferation in PC-3 prostate cancer cells

The antiproliferative effects of RE were evaluated in PC-3 cells. The androgen receptor negative PC-3 prostate cancer cells were exposed to 5, 10, 25, 50, 75, 100, or 150 µg/mL RE for 72 hours and cell proliferation was assessed using the crystal violet assay. Treatment with RE resulted in a dose–dependent inhibition of cell proliferation. A significant inhibition (80.25 ±4.44 % of control, $p<0.01$) was seen with 10 µg/mL RE while maximum inhibition (26.51 ±2.95 % of control, $p<0.0001$) was seen with 50 µg/mL RE (Figure 11A and 11B). Higher RE concentrations (75 and 100 µg/mL) did not result in a statistically greater inhibition of cell proliferation compared to 50 µg/mL (Figure 11A). The IC_{50} of RE for cell proliferation was 19.72 µg/mL. In addition, docetaxel, derived from *Taxus baccata* and paclitaxel, derived from *Taxus brevifolia,* are established medications used clinically in the treatment of prostate cancer [135] and we used them in the present study to compare the effects of RE to the effects of the aforementioned chemotherapeutic drugs. Treatment of the cells with 5 nM PTX did not result in any significant inhibition of cell proliferation (91.27 ±2.79% of control, $p>0.05$) (Figure 11B), while treatment with 10 nM of paclitaxel showed significant inhibition of cell proliferation (67.63 ±4.24% of control, $p<0.01$) (Figure 11B). Importantly, the inhibition of cell proliferation seen with 50 µg/mL of rosemary extract (26.51 ±2.95 % of control, $p<0.0001$) was greater than that seen with both 5 nM (38.11 ±1.16% of control, $p<0.0001$) and 10 nM DTX (32.08 ±0.84% of control, $p<0.0001$) treatment.

(A)



(B)

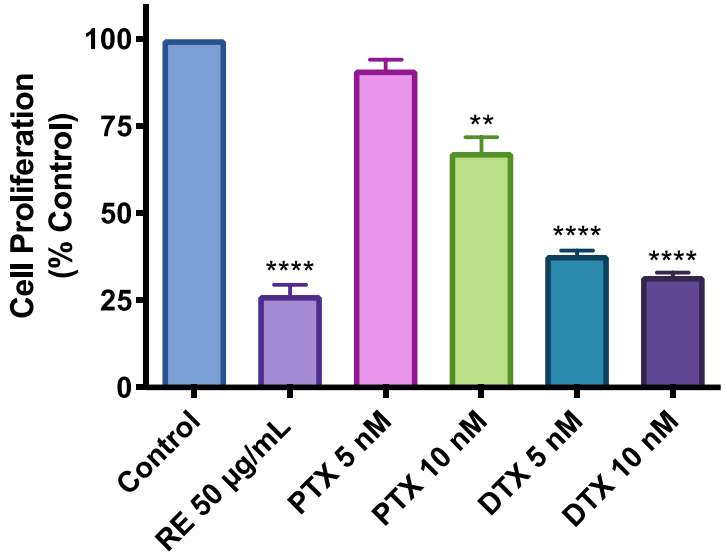
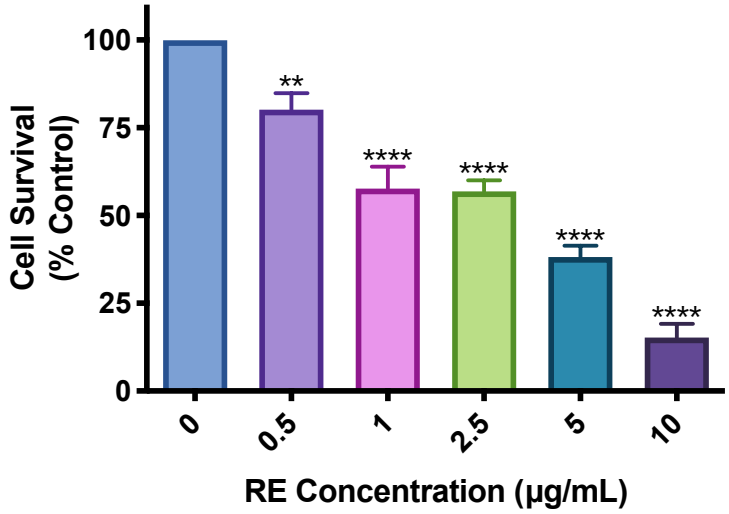


Figure 11. Effect of rosemary extract on PC-3 prostate cancer cell proliferation. PC-3 cells were treated without (control) or with the indicated concentrations of rosemary extract (RE) (A, B), paclitaxel (PTX) (B), or docetaxel (DTX) (B) for 72h followed by fixing and staining with 0.5% crystal violet. The stain was solubilized, and absorbance was read at 570 nm. Data are the mean ± SEM of 6 independent experiments. **p<0.01, ****p<0.0001.

4.2.2 Effects of RE on cell survival in PC-3 prostate cancer cells

The ability of cancer cells to survive and form colonies was also assessed in PC-3 cells using the clonogenic survival assay. Exposure of PC-3 cells to 0.5, 1, 2.5, 5, or 10 µg/mL of RE resulted in a concentration-dependent inhibition of survival (Figure 12A). The lowest concentration of RE that resulted in a significant inhibition of cell survival was 0.5 µg/mL (80.20 ±4.60% of control, $p<0.01$). The greatest inhibition (15.27 ±3.80% of control, $p<0.0001$) of cell survival was seen at 10 µg/mL RE (Figure 12A and 12B). Based on our data we calculated the RE concentration for the half maximal inhibition (IC_{50}) of cell survival was 2.43 µg/mL. Exposure of the cells to 0.5 mM of paclitaxel resulted in a significant inhibition of cell survival (64.12 ±6.94% of control, $p<0.05$). Similarly, cell survival in PC-3 cells treated with 5 nM of PTX was also significantly inhibited (23.81 ±11.92% of control, $p<0.0001$) (Figure 12B). In addition, treatment with both 0.5 nM (59.18 ±9.45% of control $p<0.01$) and 5 nM docetaxel (8.50 ±8.50% of control $p<0.0001$) resulted in a significant inhibition of cell survival. It should be noted that the inhibition of PC-3 cell survival seen with 10 µg/mL of RE (15.27 ±3.80% of control, $p<0.0001$) was similar to the inhibition seen with 5 nM docetaxel (8.50 ±8.50% of control $p<0.0001$) in PC-3 prostate cancer cells.

(A)



(B)

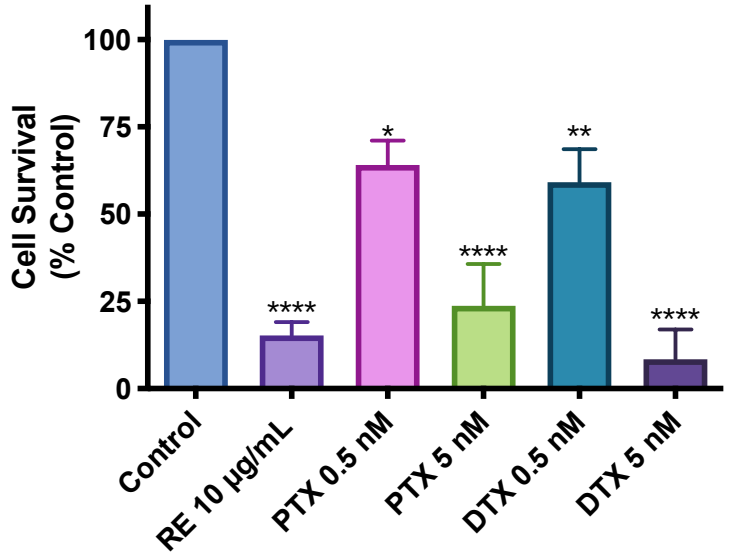
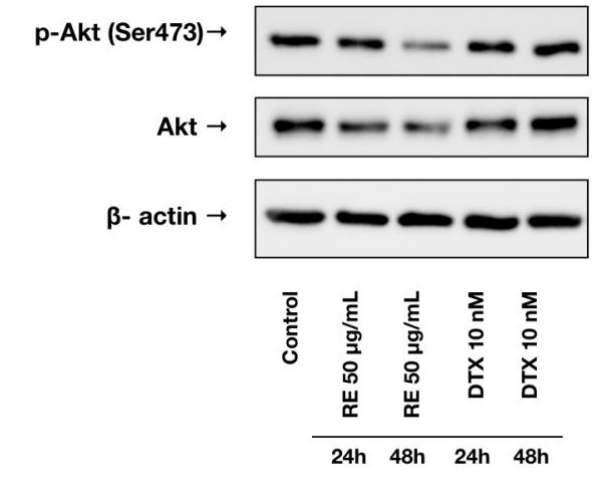


Figure 12. Effect of rosemary extract on PC-3 prostate cancer cell survival. PC-3 cells were seeded (1000 cells/well) in a 6-well plate and exposed to the indicated concentrations of rosemary extract (RE) (A, B), paclitaxel (PTX) (B), or docetaxel (DTX) (B) for 7 days followed by fixing and staining with 0.05% methylene blue. Colonies of more than 50 cells were counted. Data are the mean ± SEM of 6 independent experiments. *p<0.05, **p<0.01, ****p<0.0001.

4.2.3 Effects of RE on Akt signaling in PC-3 prostate cancer cells

We also examined the effects of RE treatment on the activation of the Akt and measured the levels of Akt phosphorylation on the serine 473 residue, an established indicator of Akt activity [131]. Treatment of PC-3 breast cancer cells with rosemary extract (50 µg/mL) for 24 hours did not result in a significant inhibition of Akt phosphorylation/activation (103.47 ±6.87% of control, $p>0.05$). However, PC-3 cells treated with RE for 48 hours (46.46 ±2.62% of control, $p<0.01$) resulted in a significant decrease in Akt phosphorylation/activation (Figure 13A and 13B). The total Akt levels were also significantly inhibited by RE treatment for 48 hours (70.37 ±6.56% of control, $p<0.01$). Treatment of PC-3 cells with docetaxel for both 24 (100.75 ±12.91% of control, $p>0.05$) and 48 hours (89.42 ±18.17% of control, $p>0.05$) did not show a statistically significant inhibition of Akt phosphorylation/activation (Figure 13B).

(A)



(B)

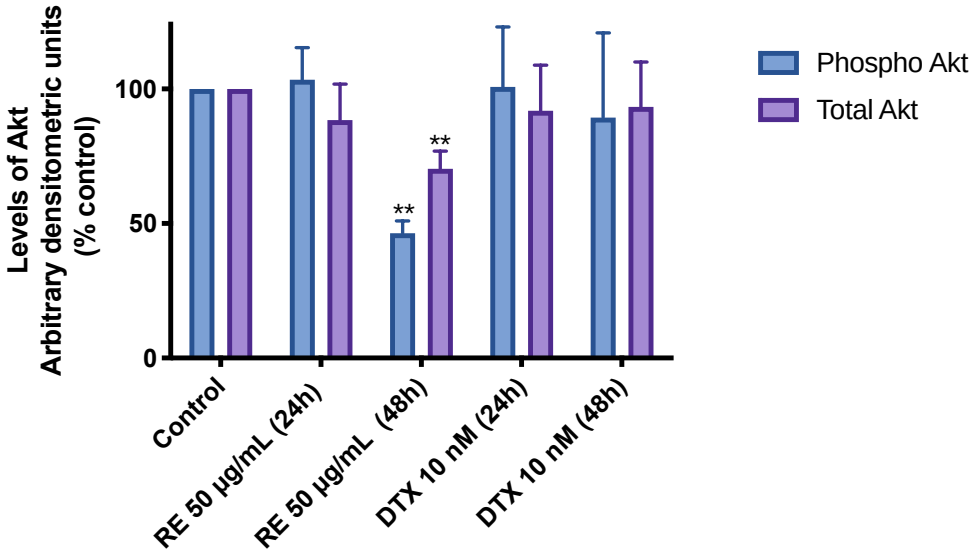
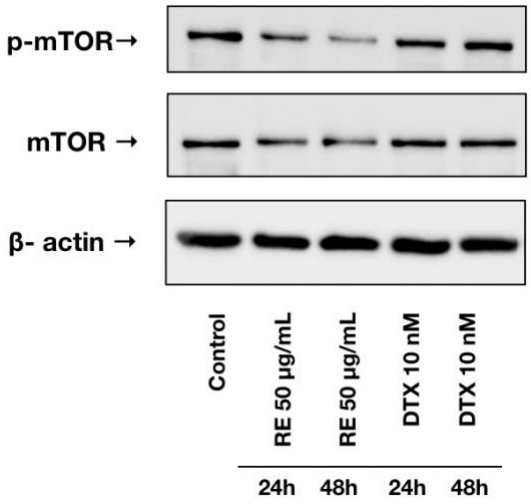


Figure 13. Effect of rosemary extract on Akt signaling in PC-3 prostate cancer cells. Cell lysates were prepared from PC-3 prostate cancer cells treated without (control) or with 50 µg/mL rosemary extract (RE) for 24 or 48 hours. Cell lysates (20 µg) were immunoblotted using specific antibodies against Akt, phosphorylated/activated Akt, or β-actin. A representative immunoblot is shown (A). The densitometry of the bands were corrected to β-actin levels and expressed in arbitrary densitometry units as % of control. The data are the mean ± SEM of 3 independent experiments. **p<0.01.

4.2.4 Effects of RE on mTOR signaling in PC-3 prostate cancer cells

Next, we examined the effect of RE on mTOR phosphorylation/activation levels. Treatment of PC-3 cells with RE for both 24 (52.92 ±10.24% of control, $p<0.01$) and 48 hours (45.57 ±9.38% of control, $p<0.001$) resulted in a significant decrease in mTOR phosphorylation (Figure 14A and 14B). Treatment with RE also resulted in a statistically significant inhibition of total mTOR levels for both 24 (74.44 ±10.86% of control, $p<0.01$) and 48 hours (66.77 ±9.25% of control, $p<0.001$). Exposing PC-3 cells to docetaxel did not result in a statistically significant inhibition of mTOR phosphorylation/activation at either 24 (94.59 ±14.70% of control, $p>0.05$) or 48 hour (91.22 ±13.90% of control, $p>0.05$) treatment time (Figure 14B).

(A)



(B)

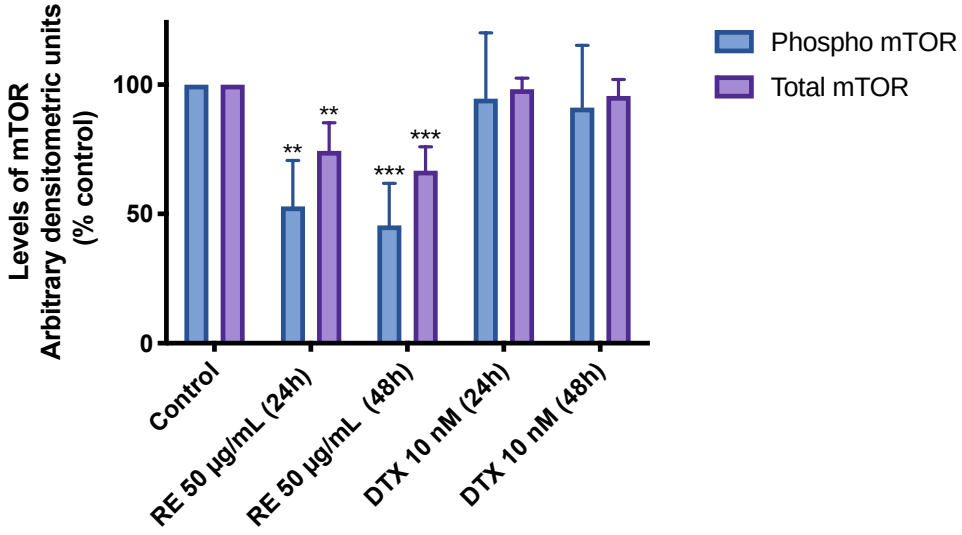
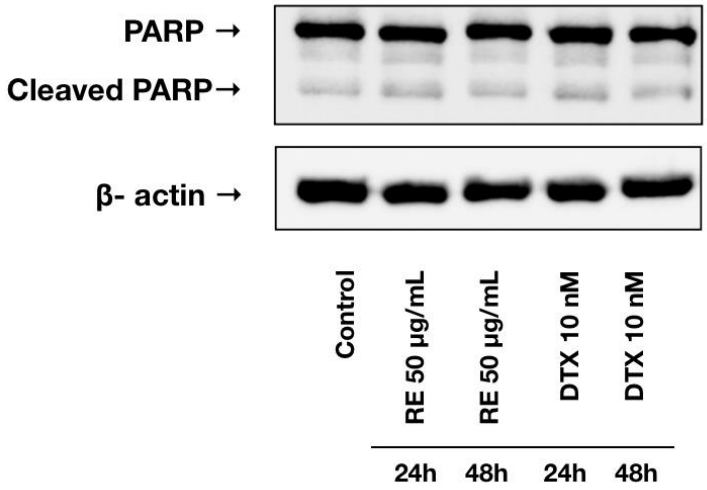


Figure 14. Effect of rosemary extract on mTOR signaling in PC-3 prostate cancer cells. Cell lysates were prepared from PC-3 cells treated without (control) or with 50 µg/mL rosemary extract (RE) for 24 and 48 hours. Cell lysates (20 µg) were immunoblotted using specific antibodies against mTOR, phosphorylated/activated mTOR or β-actin. A representative immunoblot is shown (A). The densitometry of the bands were corrected to β-actin levels and expressed in arbitrary densitometry units as % of control (B). The data are the mean ± SEM of 3 independent experiments. **p<0.01, ***p<0.001.

4.2.5 Effects of RE on apoptosis in PC-3 prostate cancer cells

The effect of RE on cell apoptosis was examined by measuring the levels of cleaved PARP, since it is an established indicator of apoptosis [80]. Exposing PC-3 prostate cancer cells to rosemary extract (50 µg/mL) for 24 hours resulted in a significant increase in the cleaved PARP/PARP ratio (177.9 ±14.50% of control, $p<0.01$) relative to the control indicating enhanced apoptosis (Figure 15A and 15B). Treatment of MDA-MB-231 cells with RE for 48 hours did not result in an increased cleaved PARP/PARP ratio (124.8 ±9.39% of control, $p>0.05$). Additionally, neither 24 (108.7 ±20.48% of control, $p>0.05$) or 48 hour (113.2 ±20.07% of control, $p>0.05$) docetaxel treatment enhanced the cleaved PARP/PARP ratio (Figure 15B).

(A)



(B)

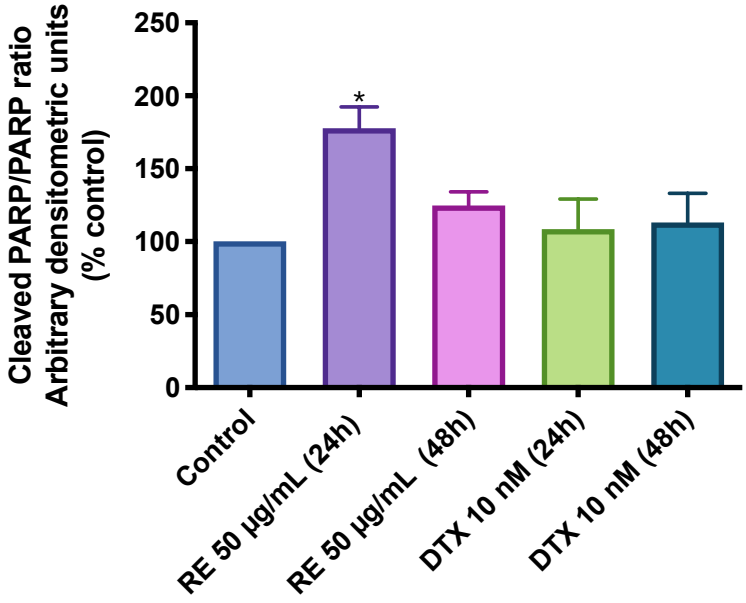


Figure 15. Effect of rosemary extract on PC-3 prostate cancer cell apoptosis. Cell lysates were prepared from PC-3 prostate cancer cells treated without (control) or with 50 µg/mL RE for 24 or 48 hours. Cell lysates (20 µg) were immunoblotted using specific antibodies against PARP or β-actin. A representative immunoblot is shown (A). The densitometry of the bands were corrected to β-actin levels and expressed as a fold increase of arbitrary densitometry units as % of control (B). The data are the mean ± SEM of 3 independent experiments. *$p<0.05$.

Figure 16 shows a representative image of control untreated, RE treated, and DTX treated PC-3 cells. No changes in cell morphology were observed with any of the treatments. It is important to note that the same number of cells were seeded in all wells. As it can be seen from Figure 16, treatment with 50 µg/mL of RE for 24 or 48 hours resulted in a substantial reduction of cell density relative to the control untreated groups. Although treatment with DTX for 24 and 48 hours showed a reduction in cell density, it was not as great as the inhbition seen with RE.

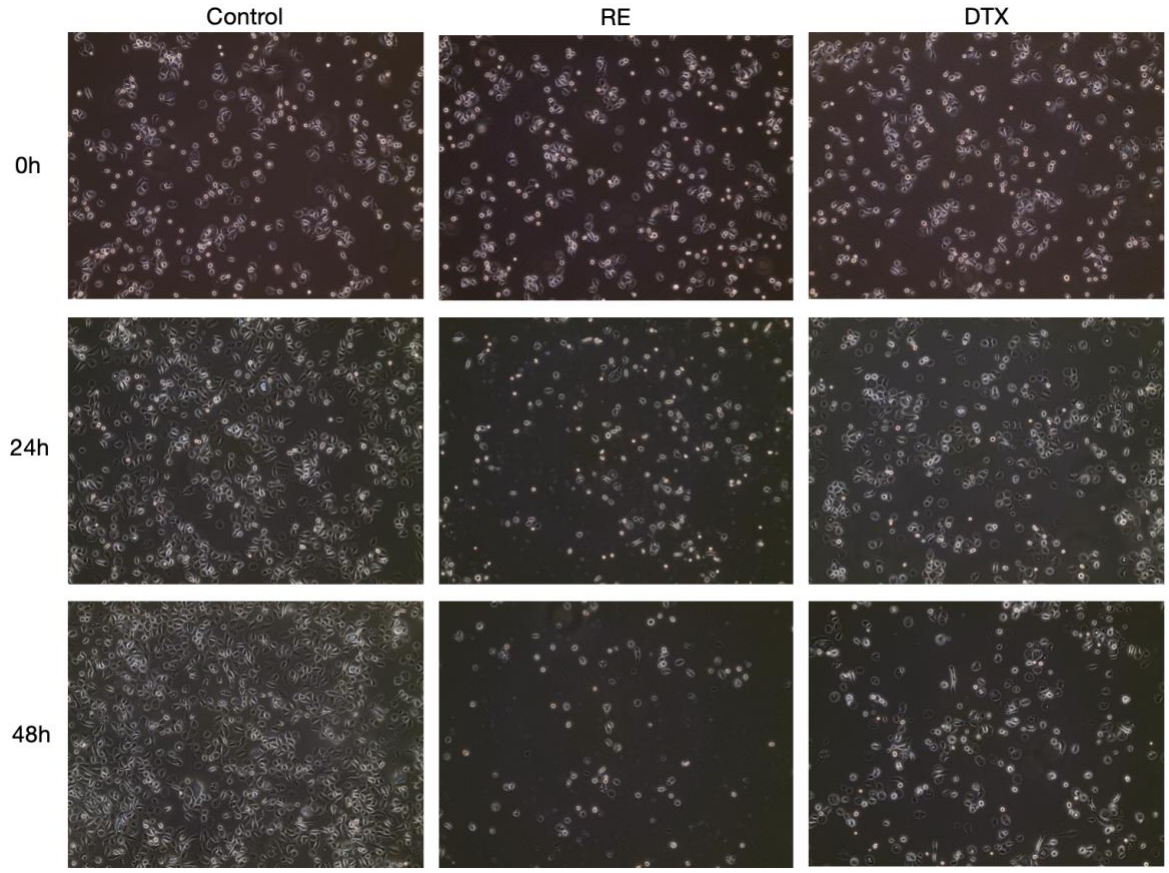
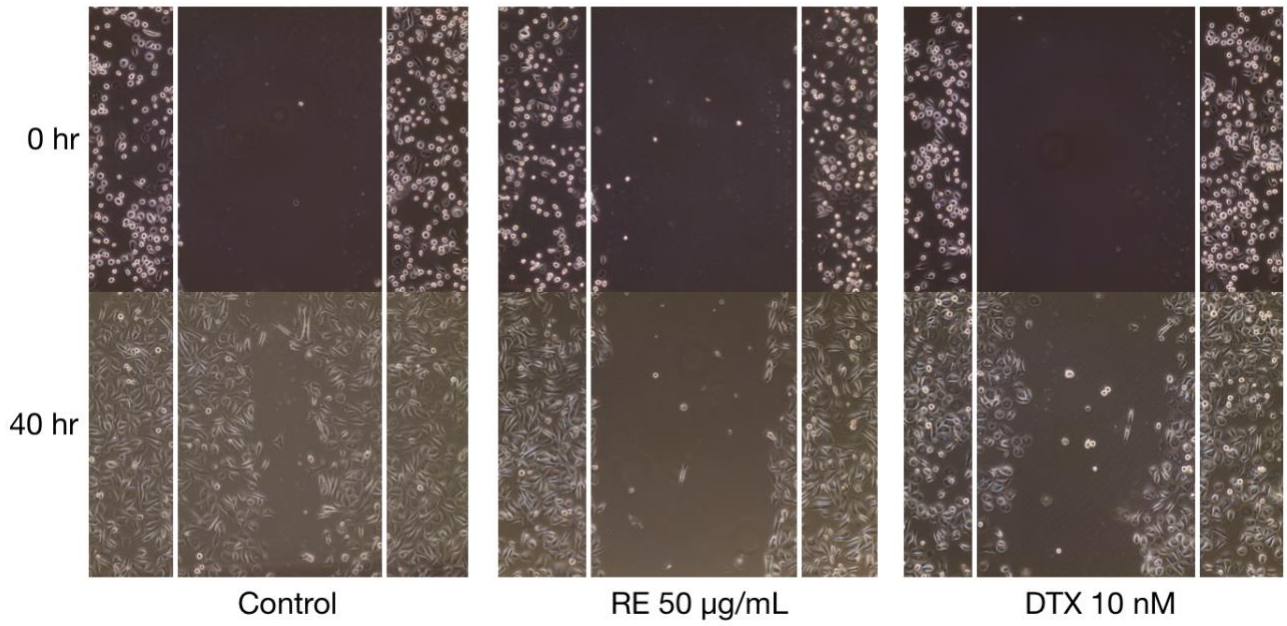


Figure 16. Effect of rosemary extract and docetaxel on PC-3 cell morphology. Cells were treated without (control) or with RE (50 µg/mL) or DTX (10 nM) for 24 or 48 hours. Photographs were taken immediately after treatment (0 hr) and after the inidctaed time points using an EVOS XL Core Cell Imaging System by Life Technologies (10X magnification).

4.2.6 Effects of RE on PC-3 prostate cancer cell migration

The wound healing assay was used to assess the ability of cancer cells to migrate. PC-3 cells were exposed to 1 µg/mL of mitomycin-C (MMC) for 1 hour to inhibit cell proliferation and were then treated without (control) or with either 50 µg/mL rosemary extract or 10 nM docetaxel for 40 hours, just before complete wound closure. Treatment with rosemary extract was shown to significantly inhibit wound closure (56.14 ±3.48% of control, $p<0.0001$) indicating properties against cell migration (Figure 10B). A significant inhibition of cell migration was also seen when treating the cells with 10 nM docetaxel (70.92 ±2.35% of control, $p<0.001$), however this inhibition was not as great as the response seen with RE (Figure 17A and 17B).

(A)



(B)

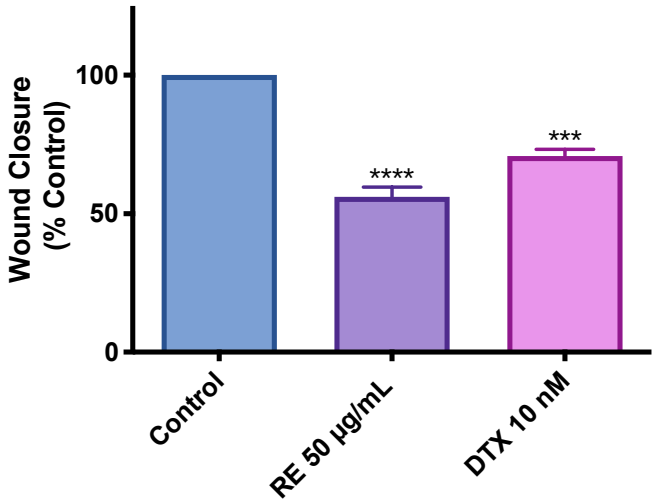


Figure 17. Effect of rosemary extract on PC-3 prostate cancer cell migration. PC-3 cells were exposed to 1 µg/mL of mitomycin-C for one hour, followed by treated without or with 50 µg/mL rosemary extract (RE) or 10 nM docetaxel (DTX) for 40 hrs. Representative images of wound healing are shown immediately after being scratched with a 200 µL pipette tip (0 hr) and after 40 hours of treatment (A). Wound closure was calculated as indicated in the methods and expressed as a percent of control untreated cells (B). The data are the mean ± SEM of 3 independent experiments. ***p<0.001, ****p<0.0001.

Table 7: Summary of the effects of RE on Akt and mTOR signaling by cell line.

Cancer Cell Line	Known Mutation Status	Total Protein Level	Phosphorylated Protein Level
MDA-MB-231	p53 null KRAS mutation	Akt ↓ mTOR↓	Akt ↓ mTOR ↓
PC-3	p53 null PTEN null	Akt ↓ mTOR ↓	Akt ↓ mTOR ↓

Chapter 5: Discussion

5.1 RE inhibits MDA-MB-231 breast cancer cell proliferation and survival

In the present study we found a dose-dependent inhibition of MDA-MB-231 breast cancer cell proliferation with rosemary extract treatment. The highest inhibition of proliferation was seen at 50 µg/mL RE (34.79 ±2.321 % of control, $p<0.0001$) with a calculated IC_{50} value of 28.86 µg/mL. Our data are in agreement with a few other studies [102,103,107,136] that have examined the effect of RE in breast cancer cells. In MCF-7 (ER+) and MDA-MB-231 breast cancer cells rosemary extract decreased cell viability in a dose-dependent manner, with an IC_{50} value of 20.42 µg /mL [102]. Similarly, rosemary extract dose-dependently decreased viability of five breast cancer cell lines including MDA-MB-231 cells [103]. This suggests that only the effect of RE on cell viability has been previously investigated in MDA-MB-231 cells, thus making our findings novel. Apart from the above mentioned two studies [102,103] that examined the effect of RE on breast cancer cell viability, Marrelli et al. found that MCF-7 (ER+) breast cancer cells treated with 100 µg/mL of RE had reduced (45% of control) proliferation [107]. A study by Telang et al. found that treating HER-2 enriched 184-B5 mammary epithelial cells with 10 µg/mL of RE inhibited cell cycle progression, inhibited the G_1 to S phase transition and induced G_1 phase arrest [136]. Collectively, these results suggest that RE may inhibit various breast cancer cells regardless of receptor status. Furthermore, the RE polyphenols carnosol, carnosic acid, and ursolic acid, effectively inhibited proliferation of ER positive MCF-7 breast cancer cells [136]. We have previously found an inhibition of A549 lung cancer cell proliferation by RE treatment [124] with an IC_{50} value of 15.9 µg/mL. Based on this evidence and comparing

the IC_{50} values it appears that TN breast cancer cells (IC_{50} 28.86 µg/mL) are less sensitive to RE treatment than lung adenocarcinoma A549 cells (IC_{50} 15.9 µg/mL) [124].

Apart from the suppression of cell proliferation, the present study found that treatment with RE inhibited clonogenic survival of MDA-MB-231 cells in a dose-dependent manner. Significant inhibition was seen at the lowest dose of 2.5 µg/mL RE (66.47 ±7.39% of control, $p<0.001$) and the highest inhibition was seen at 25 µg/mL RE (9.33 ±3.09% of control, $p<0.0001$) with a calculated IC_{50} value of 4.82 µg/mL. While no other studies have examined the effect of RE treatment on TN breast cancer cell survival, two studies using MCF-7 (ER+) breast cancer cells [103] and 184-B5/HER cells, which represent a model for the HER-2 enriched breast cancer subtype [136], found dose-dependent inhibition of survival with RE treatment. A calculated IC_{50} value of 4.6 µg/mL was found in RE treated 184-B5/HER cells. Comparing this IC_{50} of RE (4.6 µg/mL) to the one found in the present study (4.82 µg/mL) we see almost identical values suggesting that different breast cancer cells may have a similar sensitivity to RE. It is important to note that the inhibition of MDA-MB-231 cell proliferation and survival by RE treatment in the present study was greater than the inhibition seen with metformin or paclitaxel treatment. Metformin is derived from the plant *Galega officinalis*, commonly known as French lilac, used for many years in the management of type 2 diabetes mellitus [129] and many recent studies [127,128,137] have provided evidence of its anticancer properties. In previous studies by our lab, we found a significant inhibition of lung cancer cell proliferation and survival by metformin treatment [126] and for this reason we used it in the current study with the intention to serve as a positive control. Metformin (5mM) treatment did not have a significant effect on cell proliferation while it resulted in a significant inhibition of cell survival (63.36 ±6.01% of control) that was less than the inhibition seen with RE treatment. We also wished to compare the effect of

RE treatment with that of an agent used clinically to treat TN breast cancer and we used paclitaxel. Specific chemotherapeutic agents targeting TN breast cancer do not exist and this type of cancer is often treated using paclitaxel [36,130,138]. Cell viability and proliferation of TN breast cancer cells was decreased with paclitaxel treatment in previous studies [103,130]. TN breast cancer patients treated with paclitaxel have serum levels of paclitaxel in the nanomolar (12 nM) [138] to the micromolar (3.3 µM) [139] range. In the present study we found a significant inhibition of proliferation, and survival with 2-10 nM paclitaxel treatment. We used these concentrations of paclitaxel based on previous *in vitro* studies [103,130] and taking into account the paclitaxel serum levels in treated patients [138,139]. Importantly, the effects of RE treatment were greater than the effect of paclitaxel and these data suggest a strong anticancer potential of RE that justifies further studies

5.2 Exploring the effect of RE on signaling molecules involved in breast cancer

The effect of RE on signaling pathways that control MDA-MB-231 cell proliferation and survival has not been studied previously. It should be noted that the levels of phosphorylated Akt (Figure 6) in the control untreated MDA-MB-231 cells are high compared to normal cells, indicating that this may be driving proliferation and survival. Akt controls cellular growth and survival/apoptosis [140] and its activation is increased in breast cancer cells including breast cancer [3,56,140,141]. Our observation is in agreement with other studies that have shown that the levels of phosphorylated/activated Akt are high in TN breast cancer [142]. Importantly, our study is the first to show that treatment with 50 µg/mL RE significantly inhibited phosphorylation/activation of Akt (Figure 6). The recognition of enhanced Akt signaling in cancer, including breast cancer [143,144], has led to the development of novel agents targeting

Akt, such as perifosine, SF1126, PX166, BEZ256, and EX147, all currently in clinical trials [143]. RE has a potent inhibitory effect on Akt, comparable to the above mentioned Akt inhibitors, and deserves attention and further investigation.

In addition to Akt, mTOR, a signaling molecule that promotes protein synthesis, cell proliferation and survival, is often activated in cancer due to mutations that are found upstream of mTOR itself, such as a gain-of-function mutation of PI3K and loss-of-function mutations on the tumor suppressor gene PTEN [13]. The activation of mTOR increases the rate of protein synthesis and suppresses autophagy in cancer cells [145]. It has been shown that activated mTOR leads to increased tumor progression and decreased patient survival, importantly, mTOR activation has been reported to be common in TN breast cancer [145,146]. In agreement with these evidence, we observed that the phosphorylation/activation levels of mTOR in the control untreated MDA-MB-231 cells (Figure 7) were high indicating its overactivation. Our data show a very potent inhibition of mTOR with RE treatment and are clinically relevant since development of effective mTOR inhibitors are highly desired to be used in the treatment of cancer. Rapamycin (sirolimus), the first discovered mTOR inhibitor, has been shown to inhibit cell growth in several types of cancer including breast [75,145]. However, rapamycin is not widely used as a cancer monotherapy due to its poor pharmacokinetics and low solubility [145].

We observed potent inhibition of both Akt and mTOR phosphorylation/activation with RE treatment in the present study. As mentioned above, effective inhibitors of Akt, mTOR or both are highly desired and employed in the treatment of TN breast cancer [64,147]. An early phase clinical trial showed that ipatasertib, an Akt inhibitor, combined with paclitaxel improved the progression-free survival of patients with TN breast cancer compared to paclitaxel alone (6.2 vs. 4.9 months, p=0.037) [148]. Another phase II study showed that the combination of

ipatasertib (400 mg daily) and paclitaxel (80 mg/m$_2$ weekly) was well tolerated by women with TN breast cancer [63]. The mTOR inhibitor everolimus was shown to increase the effectiveness of paclitaxel in treating patients with TN breast cancer in clinical trials [64].

It is important to note that apart of the reduction in the phosphorylated/activated Akt and mTOR levels, seen with RE treatment in our study, our data showed a significant reduction in the total Akt and mTOR levels. The reduction in total Akt levels seen in our study is significant. Similar to our data, treatment with salinomycin, an antibiotic with selective activity against some cancer cells, alone or in combination with MK-2206, an allosteric Akt inhibitor, resulted in reduced phosphorylated and total Akt levels in Hs578T breast cancer cells [149]. Total mTOR protein levels are elevated in breast cancer compared to normal cells [150]. Although mTOR inhibitors have shown promise as anti-cancer agents, there is a high risk of drug resistance [150]. Inhibition of mTOR initiates a feedback loop that upregulates upstream receptor tyrosine kinases, which activate Akt, and reactivate mTOR limiting the effectiveness of these inhibitors [66,151]. This feedback regulation of mTOR involves increased levels of total-mTOR and is a suggested mechanism of drug resistance [150]. Thus, utilizing an agent that will inhibit the expression of the mTOR protein, in addition to the inhibition of mTOR activation, may potentially improve the efficacy of breast cancer treatments and decrease drug resistance [150]. Similar to our data, it was shown that MCF-7 breast cancer cells treated with metformin and rapamycin, two known mTOR inhibitors, decreased levels of both total and phosphorylated/activated mTOR [150]. Overall, utilizing agents that target Akt/mTOR and inhibit both phosphorylated and total levels of these proteins, may result in increased sensitization to treatments and reduced drug-resistance.

RE also enhanced the level of cleaved PARP (Figure 5), a well-established marker of apoptosis [80]. A previous study by our group also found a two-fold increase in PARP cleavage in A549 non-small cell lung cancer cells treated with RE [124]. Similar to our findings, treatment of MDA-MB-231 cells with carnosol, an RE polyphenol, resulted in increased apoptosis, as indicated by increased cleaved PARP levels and increased cleaved caspase 3, 8, and 9 levels [113]. Carnosol was also seen to increase the expression of the pro-apoptotic protein Bax and decrease the anti-apoptotic protein Bcl2 [113]. It is possible that the pro-apoptotic effect seen with the RE treatment in our study may be due to carnosol or other polyphenols found in RE. Previously, we have measured the levels of the polyphenols carnosic acid and rosmarinic acid in our extract and found it to be 2.12 ±0.22% [152] and 13.39 ±0.23% [153], respectively. A few *in vitro* studies have shown that carnosic acid, rosmarinic acid, and carnosol inhibited cell proliferation and induced apoptosis in various breast cancer cell lines [102,106,113,154]. Specifically, carnosic acid was shown to inhibit proliferation in ER-negative human breast cancer cells by inducing G1 cell cycle arrest [106]. Treatment with carnosic acid (19 μM) showed 70% inhibition of MCF-7 (ER+) breast cancer cell viability, while a 2-fold greater inhibition was seen in the estrogen independent TN MDA-MB-231 breast cancer cells [102]. Similarly, carnosol reduced the cell viability of MDA-MB-231 breast cancer cells in a dose-dependent manner with an IC_{50} value of 83 μM [113]. Interestingly, rosmarinic acid did not inhibit proliferation of MDA-MB-231 breast cancer cells [102] suggesting that this polyphenol may not be responsible for the anti-proliferative effects shown by RE treatment. Based on these studies we hypothesize that the polyphenols carnosic acid and carnosol may be responsible for the observed effects of RE in the present study.

There are no studies examining the effects of RE treatment using TN breast cancer xenograft models. In one study, intraperitoneal injections of RE or carnosol at 200 mg/kg for 5 days in female rats inhibited the DMBA-induced mammary adduct formation (44% and 40% respectively) [115]. In another study, administration of carnosic acid to mice inoculated with ER positive breast cancer cells resulted in significant inhibition of tumor growth [109]. Furthermore, treatment of mice xenografted with ER positive breast cancer cells with carnosic acid and tamoxifen (30 and 10 mg/kg, respectively) as a combined therapy resulted in a greater inhibition of tumor growth in comparison to carnosic acid or tamoxifen monotherapy [109]. Although the studies examining the effects of rosemary extract and rosemary extract polyphenols *in vivo* are limited, the available evidence suggest that they may be effective in inhibiting tumor growth as a monotherapy or as a combined therapy with other chemotherapeutic agents. *In vivo* animal studies utilizing TN breast cancer xenograft models are required to better understand the effects of RE and RE polyphenols against this subtype of cancer.

5.3 RE inhibits PC-3 prostate cancer cell proliferation and survival

The present study found a dose-dependent inhibition of PC-3 prostate cancer cell proliferation with rosemary extract treatment. The highest inhibition was seen at 50 µg/mL (26.51 ±2.95% of control, $p<0.0001$) RE with an IC_{50} 19.72 µg/mL. A significant inhibition of cell proliferation was first measured at 10 µg/mL of rosemary extract (80.25 ±4.44% of control, $p<0.01$). Our data are in agreement with other studies [102,117] that have examined the effect of RE in prostate cancer cells. Similar to our findings treatment with RE decreased proliferation of 22RV1 (76.5%) and LNCaP (94.6%) prostate cancer cells [117]. Cell viability was also decreased in a dose-dependent manner, the observed IC_{50} values for 22RV1 and LNCaP cells

was 13.3 µg/mL and 27 µg/mL, respectively [117]. Another study showed that RE dose-dependently decreased cell proliferation in various human cancer cell lines including DU145 (human prostate carcinoma) cells [102]. Furthermore, the RE polyphenol carnosic acid was shown to significantly decrease cell proliferation in PC-3, LNCaP, DU145, and 22RV1 prostate cancer cells in various studies [118–120]. When comparing the calculated IC_{50} values it appears that PC-3 cells (19.72 µg/mL) are more sensitive to RE treatment than breast adenocarcinoma MDA-MB-231 cells (28.86 µg/mL) shown in this study, but less sensitive than lung adenocarcinoma A549 cells (IC_{50} 15.9 µg/mL) [124].

Apart from cell proliferation, the effects of RE on cell survival were also examined in PC-3 cells. A highest inhibition of cell survival was seen with 10 µg/mL RE treatment (15.27 ±3.80% of control, $p<0.0001$), with a calculated IC_{50} value of 2.43 µg/mL. A significant inhibition of cell survival was seen even when using RE concentrations as low as 0.5 (80.20 ±4.60% of control, $p<0.01$) and 1 µg/mL (57.73 ±6.19% of control, $p<0.0001$). It is important to note that the inhibition of PC-3 cell survival seen with 10 µg/mL RE (15.27 ±3.80% of control, $p<0.0001$) was similar to the inhibition seen with 5 nM DTX (8.50 ±8.50% of control, $p<0.0001$) treatment and greater than the inhibition seen with 5 nM PTX (23.81 ±11.92% of control, $p<0.0001$). Another study found that the rosemary polyphenol rosmarinic acid (200 µM), inhibited PC-3 and DU145 prostate cancer cell survival [155]. This suggests that RA may play a role in the inhibition of PC-3 cell survival seen with rosemary extract. Based on the IC_{50} values determined using the findings from our study examining the inhibitory effect of RE on cell survival, PC-3 cells (2.43 µg/mL) appear to be more sensitive to rosemary extract treatment than MDA-MB-231 cells (4.82 µg/mL).

5.4 Exploring the effect of RE on signaling molecules involved in prostate cancer

The effects of RE on Akt and mTOR signaling were examined in PC-3 prostate cancer cells. Our study showed a significant inhibition of Akt phosphorylation/activation (46.46 ±2.62% of control, $p<0.01$) with 50 µg/mL RE treatment for 48 hours (Figure 13). Total Akt values were also significantly reduced during 48 hour treatment (70.37 ±6.56% of control, $p<0.01$), similar to our findings in MDA-MB-231 cells. Cells treated with RE for 24 hours did not result in any inhibition of phosphorylated/activated Akt (103.47 ±6.87% of control, $p>0.05$). Phosphorylated levels of Akt were also not significantly decreased by either 24 hour (100.75 ±12.91% of control, $p>0.05$) or 48 hour (89.42 ±18.17% of control, $p>0.05$) DTX treatment. There are no other studies examining the effects of RE on Akt signaling in PC-3 prostate cancer cells, thus making our findings novel. However, a previous study by our group treated A549 non-small cell lung cancer (NSCLC) cells with 50 µg/mL RE and also found a significant inhibition of Akt phosphorylation/activation (36.1 ±4.9% of control, $p<0.001$) [124]. mTOR is a downstream target of Akt and therefore, the effects of RE were also examined on this signaling molecule. Treatment with RE for 24 hours decreased mTOR phosphorylation/activation in PC-3 cells (52.92 ±10.24% of control, $p<0.01$). Interestingly, total mTOR levels were also decreased with 24 hour RE treatment (74.44 ±10.86% of control, $p<0.01$). A significant inhibition of mTOR phosphorylation/activation (45.57 ±9.38% of control, $p<0.001$) was also seen with 48 hour RE treatment (Figure 14). In accordance with our findings in MDA-MB-231 cells, PC-3 cells treated with RE for 48 hours had decreased total mTOR levels (66.77 ±9.25% of control, $p<0.001$). Also, 24 hour RE treatment increased the cleaved PARP/PARP ratio in PC-3 cells (177.9 ±14.50% of control, $p<0.01$), while 48 hour treatment showed a tendency to enhance cleaved

PARP but was not significant (124.8 ±9.39% of control, $p>0.05$) (Figure 15). Our study did not examine the effect of rosemary extract on p70S6K, a downstream target of mTOR, in either MDA-MB-231 or PC-3 cells. However, it was previously shown by our group that RE significantly inhibited both activated (57.2 ±14.8% of control, $p<0.05$) and total (83.3 ±2.5% of control, $p<0.05$) p70S6K levels in A549 NSCLC cells [124]. Therefore, RE may also inhibit p70S6K in MDA-MB-231 breast and PC-3 prostate cancer cells, however future studies must be performed in order to confirm this.

The PI3K/Akt signaling pathway is commonly aberrant in metastatic prostate cancer [156]. Multiple Akt inhibitors are currently in various stages of clinical development [63,157]. These inhibitors fall into two main classes: ATP-competitive inhibitors and allosteric inhibitors of Akt [63,157,158]. Additionally, the loss of the tumor suppressor gene PTEN, as seen in PC-3 cells is associated with a poor prognosis in patients [156]. A phase II clinical study examined the effects of ipatasertib, an Akt inhibitor, combined with the anti-androgen medication abiraterone acetate, in patients with metastatic castration-resistant prostate cancer [159]. The combined treatment of ipatasertib and abiraterone acetate showed trends toward improved progression-free survival [159]. Importantly, in patients with PTEN loss tumors, measures of anti-tumor activity were greater with ipatasertib and abiraterone acetate combined treatment. This suggests that PTEN loss tumors may benefit greatly from Akt and AR inhibition.

In addition, it was found that the dual PI3K/Akt and mTOR inhibitor, NVP-BEZ235 (40 mg/kg), when used as a combined therapy with paclitaxel (4 mg/kg) resulted in a greater inhibition of tumor growth in castrated mice xenografted with C4-2AT6 prostate cancer cells than monotherapy treatments [160]. It is also suggested that the inhibition of PI3K/Akt/mTOR

signaling by NVP-BEZ235 can overcome docetaxel resistance in human castration resistant prostate cancer [160]. A study by Millis et al. analyzed solid tumor samples in 19,784 patients with over 40 types of cancer and identified aberrations in the PI3K/Akt/mTOR pathway in 38% of samples, indictaing an urgent need to find chemicals that can target signaling molecules involved in this pathway [161]. In our study RE has shown anti-proliferative activity, and an inhibition of Akt and mTOR in both MDA-MB-231 breast cancer and PC-3 prostate cancer cells, suggesting that with further research RE may have potential as a chemotherapeutic agent.

The inhibition of total Akt and mTOR levels with RE treatment (Figures 13 and 14) suggests that this reduction could be due to the inhibition of gene transcription, inhibition of protein synthesis, or increased protein degradation. RE may also have an effect on protein stability. The inhibition of total Akt protein and mRNA levels has previously been reported in K562 leukemia cells treated with 50 µg/mL for 24 to 48 hours [162]. The same study also found that terpinolene, a constituent of rosemary and sage, but not COH, RA, or linalool, among some of the main components of rosemary tested, reduced Akt protein expression in K526 cells [162]. Additionally, a previous study by our group showed an significant reduction of both total Akt and mTOR expression by 50 µg/mL RE treatment in A549 lung cancer cells [124]. It is important to note that the inhibitory effects of rosemary extract on Akt and mTOR observed in the present study are similar to the effects of perifosine, a novel inhibitor of Akt, currently in clinical trials. Perifosine inhibited Akt phosphorylation and reduced total Akt levels in H157, H460, and A549 NSCLC cells [163]. Similarly, in HCT116 human colon cancer cells perifosine inhibited Akt phosphorylation and reduced total Akt, mTOR, and p70S6K levels resulting in the induction of apoptosis and autophagy [164].

5.5 RE inhibits MDA-MB-231 breast and PC-3 prostate cancer cell migration

Treatment with rosemary extract (50 µg/mL) showed a significant inhibition of MDA-MB-231 cell migration (65.15 ±0.97% of control, $p<0.001$) that was comparable to the response seen with 10 nM paclitaxel (78.44 ±5.60% of control, $p<0.01$) (Figure 10). Similarly, RE treated PC-3 cells also showed a significant inhibition of cell migration (56.14 ±3.48% of control, $p<0.0001$) that was comparable to response seen with docetaxel (70.92 ±2.35% of control, $p<0.001$) (Figure 17). The inhibitory effects of RE on cell migration have been tested in HGUE-C-1, HT-29, and SW480 human colon cells [165]. Treatment with 30 and 40 µg/mL RE significantly inhibited cell migration in all the aforementioned cell lines [165]. Although no other studies exist examining the anti-migratory or anti-metastatic effects of RE in TN breast or prostate cancer cells, one study found a significant inhibition of MDA-MB-231 cell migration when treated with the rosemary polyphenol carnosic acid [154]. In addition, the inhibition of cell migration seen with carnosic acid in combination with trastuzumab, a chemotherapeutic agent used in clinical practice, resulted in a greater inhibition of cell migration when compared to each agent alone as a monotherapy [154]. These data indicate a chemo-sensitive effect of RE polyphenols.

5.6 Limitations and future directions

One limitation of this study is that it was performed *in vitro* using a cell culture model and has limited applicability and generalizability physiologically, towards human models of cancer. However, cell culture is a well-established model for exploring the anti-cancer effects of novel compounds to determine their potential use *in vivo* and to establish concentrations that are effective at the cellular level. The major advantage of using cell culture is the consistency and

reproducibility of results and that the cellular environment can be easily manipulated to examine different conditions. Importantly, *in vitro* studies using human cancer cells provide the opportunity to examine cell signaling questions, perform experiments by over-expressing or eliminating the expression of a specific protein and find potential drug targets. Following studies *in vitro, in vivo* xenograft studies must be performed and if the data are promising, clinical human studies should follow. When the mechanism of the anti-cancer effects of RE have been well established *in vitro,* studies using *in vivo* xenograft models of breast and prostate cancer in mice should be performed. These studies can further examine effective doses and the bioavailability of RE to support its use in human clinical trials.

Another limitation of the present study is the lack of studies using healthy, non-cancerous breast and prostate cancer cells. Future studies should examine the effects of RE in normal breast and prostate epithelial cells to see if our extract can specifically target cancer cells while sparing normal healthy cells. Cell lines with no known mutations such as the mammary epithelial cell line 184B5 and the prostate epithelial cell line PNT1A should be used to compare the effects of RE in cancer cells to non-cancerous cells.

In the present study only one cell line representing TN breast cancer and prostate cancer was used and this presents another limitation. In the future, other cancer cell lines representing various subtypes of breast cancer such as those that express hormone receptors (MCF-7), HER-2 (BT474), and other TN breast cancer cell lines (MDA-MB-436) should also be examined. Additionally, various prostate cancer cell lines that are androgen receptor responsive (LNCaP) and other androgen receptor negative (DU145) cell lines should also be used to further support the anti-cancer effects of RE *in vitro*.

Importantly, it is unclear whether RE treatment is causing cell death (cytotoxic effects) or

inhibiting cell growth/proliferation (cytostatic effects) in MDA-MB-231breast and PC-3 prostate cancer cells. Future studies should use a second validation of apoptosis; such as trypan blue dye or propidium iodide staining in order to determine what the exact effect of rosemary extract is in cancer cells.

In this study, we chose to examine the effects of RE on the Akt and mTOR signaling molecules because these are often mutated in cancer and thus lead to increased cell proliferation and an inhibition of apoptosis. Cancer signaling is very complex and therefore, it would be beneficial to look at other signaling molecules involved in regulating cell proliferation and survival. Future studies should explore other signaling molecules involved in regulating proliferation and survival/apoptosis by Western blotting. Since the effects of RE on the signaling molecules involved in cell proliferation and survival varied between the two cell lines it would be interesting to use inhibitors to knockdown the expression of Akt and examine how this alters the effects seen by RE treatment.

Combination therapies are useful in treating cancer because they can target different signaling pathways at once and decrease chemo-resistance. Future studies should investigate the effect of RE in combination with current chemotherapeutic agents such as paclitaxel, docetaxel, or other natural compounds that have shown anti-cancer activity such as resveratrol.

A plant extract was used in the present study which may contain numerous bioactive compounds accounting for the observed effects. Finding these bioactive compounds should be the focus of future studies. Certain polyphenols, such as carnosic acid, carnosol and rosmarinic acid, contained within rosemary extract may play a role. Pilot studies in our lab indicate that carnosol and carnosic acid but not rosmarinic acid have anti-proliferative (Figure s3) and anti-survival effects.

5.7 Summary/Conclusions

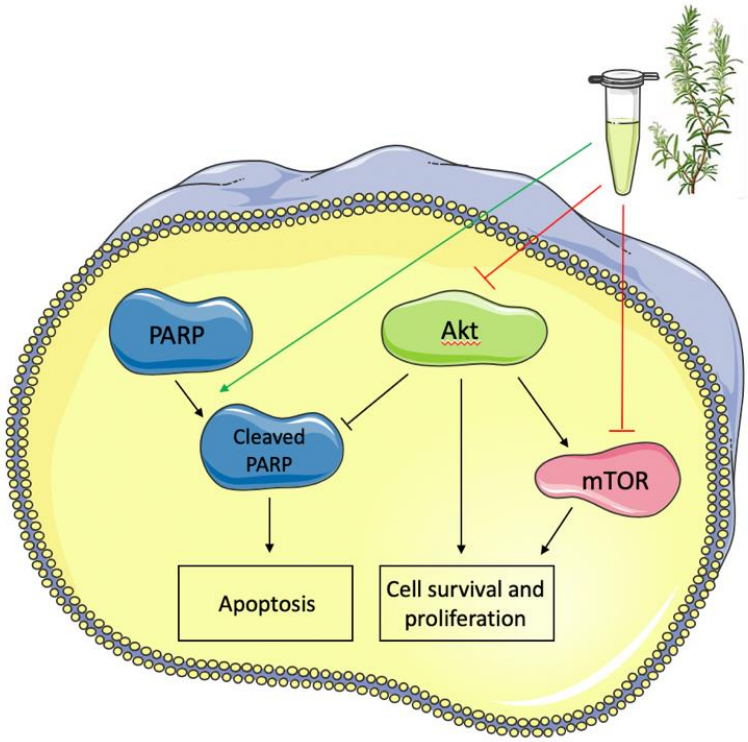


Figure 18. Effects of rosemary extract on MDA-MB-231 triple negative breast cancer and PC-3 prostate cancer cell signaling molecules. RE inhibited the phosphorylation/activation and total levels of Akt and mTOR, while enhancing the levels of cleaved PARP.

Breast cancer is the most frequently diagnosed and the second leading cause of cancer-related death among women in North America. Similarly, prostate cancer is the most commonly diagnosed type of cancer and second leading cause of cancer-related death among men in North America. Thus, both breast and prostate cancer cell lines were used in this study to represent the most common types of cancer found in patients. Triple negative breast cancer is one of the most aggressive subtypes of breast cancer and does not respond to conventional hormone therapy. Prostate cancers that are not responsive to hormone therapy display increased metastatic potential and are highly aggressive, therefore innovative treatments are being sought for both of

these types of cancer. Rosemary extract has a high polyphenolic content and has been previously shown to have antioxidant, anti-inflammatory, anti-microbial, anti-diabetic, and anti-cancer effects. This study aimed to examine the anti-cancer effects of RE in TN breast cancer cells and androgen receptor negative prostate cancer cells, as well as the potential underlying signaling mechanisms involved.

The present study is one of the few studies that has examined the effects of rosemary extract on MDA-MB-231 triple negative breast cancer and PC-3 prostate cancer cells. We found that RE significantly inhibited MDA-MB-231 and PC-3 cell proliferation, and survival at significantly low concentrations (0.5-20 µg/mL). The levels of cleaved PARP, an established marker of cellular apoptosis, were increased by RE treatment for 24 and 48 hours in MDA-MB-231 cells however, cleaved PARP was only increased with 24 hour RE treatment in PC-3 cells, suggesting that the growth inhibitory effect may vary by cell line. Importantly, RE significantly reduced the phosphorylation/activation levels of Akt and mTOR, key players controlling cancer cell proliferation and survival. These effects were comparable to the effects of paclitaxel and docetaxel, chemotherapeutic agents that are currently used in the treatment of TN breast cancer and prostate cancer.

Our data indicate that RE may have a role as a potential chemopreventive or chemotherapeutic agent and could act as a dual Akt and mTOR inhibitor. Further research should investigate a) the exact polyphenolic constituent(s) of rosemary extract that contribute to its anti-cancer effects, b) prostate and mammary epithelial cell lines representing healthy/non-cancerous cells should be used for comparison and c) the effects of RE and RE polyphenols should be explored further using *in vivo* animal models xenografted with triple negative breast cancer cells and androgen receptor negative prostate cancer cells.

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Appendix

Supplemental Figures:

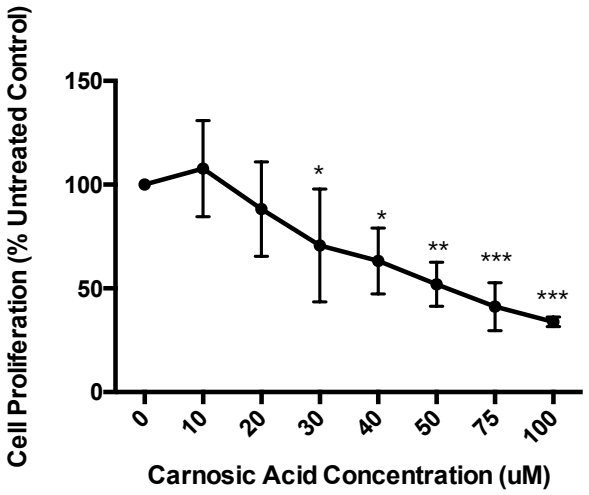

Figure s1. Effect of CA on MDA-MB-231 breast cancer cell proliferation. MDA-MB-231 cells were treated without (control) or with the indicated concentrations of carnosic acid (CA) for 72h followed by fixing and staining with 0.5% crystal violet. The stain was solubilized, and absorbance was read at 570 nm. Data are the mean ± SEM of 3 independent experiments. *p<0.05, **p<0.01,***p<0.001.

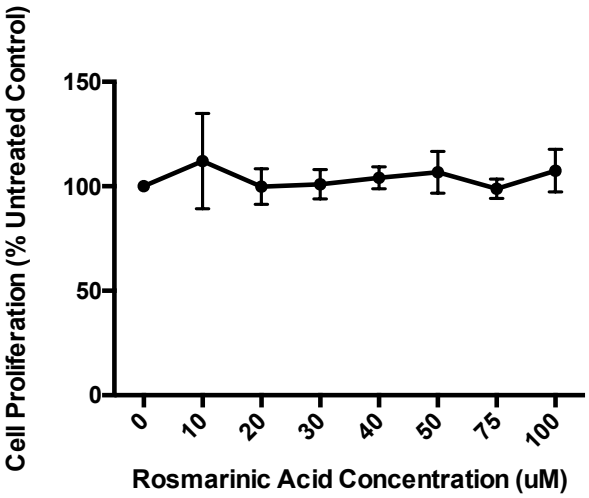

Figure s2. Effect of RA on MDA-MB-231 breast cancer cell proliferation. MDA-MB-231 cells were treated without (control) or with the indicated concentrations of rosmarinic acid (RA) for 72h followed by fixing and staining with 0.5% crystal violet. The stain was solubilized, and absorbance was read at 570 nm. Data are the mean ± SEM of 3 independent experiments.

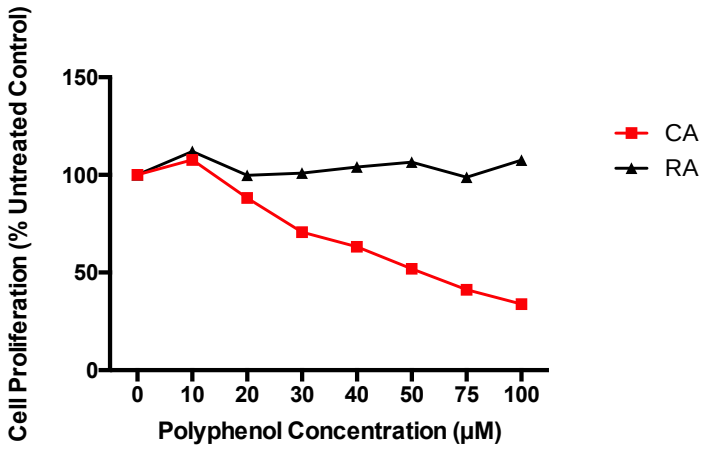

Figure s3. Comparing the effects of CA and RA on MDA-MB-231 breast cancer cell proliferation. MDA-MB-231 cells were treated without (control) or with the indicated concentrations of either carnosic acid (CA) or rosmarinic acid (RA) for 72h followed by fixing and staining with 0.5% crystal violet. The stain was solubilized, and absorbance was read at 570 nm. Data are the mean ± SEM of 3 independent experiments.